

Original Research Article

Anti-aging activities of *Pyrus pyrifolia* var *cultata* plant callus extract

Hyun Jung Kim and Jin Woo Park*

College of Pharmacy and Natural Medicine Research Institute, Mokpo National University, Muan-gun, Jeonnam, Republic of Korea

*For correspondence: **Email:** jwpark@mokpo.ac.kr; **Tel:** +82 61 450 2704; **Fax:** +82 61 450 2689

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Abstract

Purpose: To investigate the anti-aging properties of callus extract from *Pyrus pyrifolia* var. *cultata* with skin regeneration and lightening activities.

Methods: Callus extract of *P. pyrifolia* var. *cultata* was produced on Murashige and Skoog (MS) medium supplemented with picloram, followed by extraction of the callus biomass in phosphate-buffered saline. The anti-oxidant activity of the extract was assessed using the DPPH free radical-scavenging assay. Inhibition of skin pigmentation by callus extract was evaluated by measuring the melanogenesis of melanoma cells. The skin-regenerating efficacy of the extract was evaluated in terms of its ability to promote procollagen synthesis and fibroblast cell proliferation.

Results: The callus extract, at 10 mg/mL, exhibited 78.7 % free radical-scavenging activity, equivalent to that exhibited by 500 μ M ascorbic acid. The 1 mg/mL extract solution afforded a 1.4-fold greater reduction in melanocyte melanin than did a 1 mg/mL arbutin solution. Fibroblast cells treated with 5 mg/mL callus extract exhibited 127 % higher levels of proliferation and 2.0-fold higher levels of procollagen type I C-peptide synthesis, respectively, compared with the untreated controls.

Conclusion: These findings suggest that the callus extract of *P. pyrifolia* var. *cultata* may be useful as a source of anti-aging cosmetic components with skin lightening and regenerating activities.

Keywords: *P. pyrifolia* var. *cultata*, Anti-aging, Callus extract, Skin lightening, Anti-tyrosinase activity, Melanogenesis, Wound recovery, Procollagen type I C-peptide synthesis

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INTRODUCTION

Plant extracts and plant-derived compounds, such as alkaloids, saponins, carotenoids, anthocyanins, and polyphenols, are considered important active ingredients in the fields of pharmacy, food, and cosmetics [1–3]. Recently, plant tissue cultures, including callus cultures, have been suggested as useful alternatives to entire plants for preparing bioactive secondary metabolites under well-controlled conditions [4]. Callus culture is not affected by seasonal variations or geographical circumstances [5]. In

addition, plant-derived active ingredients such as polyphenols have been used to prevent skin aging; the materials protect the epidermis from external stimuli and oxidative stress, such as reactive oxygen species (ROS), chemical hazards, and solar UV radiation. However, the skin maintains the flexibility required to ensure tissue regeneration [6–8].

Pyrus pyrifolia var. *cultata*, commonly known as the Korean pear, is an important fruit crop in Asia and an excellent source of amino acids, sugars, and vitamin C [9]. The pear exhibits various physiological activities and contains valuable

compounds including flavonoids and phenolics [10]. Thus, it has been used as a traditional medicine for asthma, hypertension, and polycyclic aromatic hydrocarbons-related diseases [11,12]. It is also known to contain pectin, arbutin, and chlorogenic acid as major phenolic constituents in the fruit skin [13]. An aqueous ethanolic extract of *Pyrus pyrifolia* var. *culta* bark was found to exhibit inhibitory activity against protein glycation and anti-oxidant activity, suggesting a possible role in targeting aging and diabetic complications [14].

However, there is no report regarding the biological activities of *Pyrus pyrifolia* var. *culta* callus in skin lightening or regeneration efficacy. Therefore, we prepared callus extract from *Pyrus pyrifolia* var. *culta* and assessed its 2,2-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity as well as its inhibitory effects on tyrosinase activity and melanin synthesis. We also evaluated the skin regenerating efficacy of *Pyrus pyrifolia* var. *culta* callus extract by measuring procollagen synthesis and the proliferation of human fibroblast cells.

EXPERIMENTAL

Materials

Murashige and Skoog (MS) medium, picloram, 2,4-dichlorophenoxy-acetic acid (2,4-D), and 6-benzyladenine (BA) were obtained from Duchefa Biochemie B.V. (Haarlem, The Netherlands). Recombinant human transforming growth factor- β (rhTGF- β), L-tyrosine, L-3,4-dihydroxyphenyl-alanine (L-DOPA), tyrosinase, arbutin, ascorbic acid, and 2,2-diphenyl-2-picrylhydrazyl (DPPH) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Other solvents and chemicals were obtained from Merck KGaA (Darmstadt, Germany) and Thermo Fisher Scientific Inc. (Waltham, MA, USA).

Preparation and characterization of callus extract

The seeds of *Pyrus pyrifolia* var. *culta* were immersed in 70 % aqueous ethanol solution for 90 s, followed by surface sterilization with 2 % sodium hypochlorite solution for 30 min and rinsed with sterile double-distilled water. The seeds were then grown aseptically in MS basal medium supplemented with 3 % sucrose and 0.3 % gelrite. The cotyledons (1.0–1.5 cm) of 7-day-old germinating seeds were explanted and cut into small pieces for callus induction. The cotyledons were cultured in MS medium supplemented with 3 % sucrose, 0.3 % gelrite, and growth regulators – picloram (2 mg/L), 2,4-D

(2 mg/L), and BA (2 mg/L) alone, or in combination – to initiate callus formation. After 4 weeks, the calli were separated from the explants and placed into fresh medium of the same composition. The calli were incubated in a growth chamber under a 16-h light (25 ± 1 °C)/8-h dark (16 ± 1 °C) cycle; light at an intensity of 40 $\mu\text{M}/\text{m}^2/\text{s}$ was delivered by cool-white fluorescent tubes. After four subcultures (each of 28 days), well-grown calli that had developed from cotyledons were further cultured in MS liquid medium with 3 % sucrose, 0.3 % gelrite, and 2 mg/L picloram, with stirring at 100 rpm.

Calli were collected from 4-week-old leaf explant cultures and washed extensively with distilled water. After freeze-drying, the material was pulverized using a mortar and pestle. An aqueous extract was prepared by boiling the dried powder (200 g) in 1 L phosphate-buffered saline (PBS, pH 7.4) at 95 °C for 2 h and filtering through filter paper. A callus solution was prepared by adding 200 g callus powder to 1 L PBS (pH 7.4) at room temperature for 2 h and filtering through filter paper.

Then, the major chemical constituents of the callus extract were identified. Total phenolic content was measured using the Folin-Ciocalteu reagent, employing the procedure of Singleton and Rossi [15]. The callus solution or extract (400 μL) was oxidized in 2 mL 10 % (v/v) Folin-Ciocalteu reagent dissolved in distilled water. After 5 min at room temperature, 1.6 mL 7.5 % (w/v) sodium carbonate were added to neutralize the solution. The mixture was then incubated at room temperature for 30 min, and the absorbance of the (blue) colored solution at 765 nm was compared with that of a solution prepared using water instead of the callus solution or extract. The total phenolic content was expressed as mg gallic acid equivalents/g dry extract using a calibration curve prepared using a gallic acid solution.

The total flavonoid content was assessed using the colorimetric method described by Lamaison and Carnat, with minor modifications [16]. A 1 % (w/v) callus solution or extract (2 mL) was mixed with 2 mL 2 % (w/v) AlCl_3 . After incubation at room temperature for 15 min, the absorbance of the reaction mixture was measured at 415 nm. Quantification was performed based on a standard curve derived using quercetin, and the results were expressed as mg quercetin equivalents/g dry extract.

The radical-scavenging activity of the *Pyrus pyrifolia* var. *culta* callus aqueous solution or extract was measured using the DPPH assay.

First, the solution and extract were further diluted with distilled water and filtered through polyvinylidene fluoride membrane filters (0.45 μm in pore size). One hundred microliters of 0.2 mM DPPH in methanol were added to 100 μL of each sample solution (ascorbic acid or *Pyrus pyrifolia* var. *culta* callus aqueous solution or extract), and the mixtures were allowed to react in the dark at room temperature for 30 min. The negative control featured addition of 100 μL DPPH solution to 100 μL methanol; *Pyrus pyrifolia* var. *culta* callus aqueous solution or extract served as the positive control. The absorbance at 517 nm was measured using methanol as the blank, and the percentage antioxidant activity was calculated using the following equation: % scavenging capacity = [absorbance of the negative control at 517 nm – (absorbance of the sample at 517 nm – absorbance of the positive control at 517 nm)/absorbance of the negative control at 517 nm] \times 100.

Inhibition of tyrosinase activity and DOPA oxidation

To evaluate the effects of *Pyrus pyrifolia* var. *culta* callus solution or extract on mushroom tyrosinase activity, 100 μL of different concentrations from each sample solution (0.5–10 mg/mL of the callus solution or extract in 0.1 M PBS (pH 6.8) was mixed with 290 μL of 0.1 M PBS (pH 6.8) and 100 μL of substrate (10 mM of L-tyrosine or L-DOPA). As positive controls, 0.5–10 mg/mL of arbutin or ascorbic acid in 0.1 M PBS (pH 6.8) were also tested. After incubation at room temperature for 5 min, 10 μL of mushroom tyrosinase (2500 units/mL in PBS) was added to initiate the reaction. Then, the assay mixture was incubated at 37 $^{\circ}\text{C}$ for 10 min. Absorbance of the reaction mixture at 475 nm was recorded and the percentage inhibition of L-tyrosine or L-DOPA oxidation was calculated as follows: % inhibition = 100 – (B/A \times 100), where A = absorbance at 475 nm in 10 min without a sample and B = absorbance at 475 nm in 10 min with test sample.

Determination of inhibition of melanin biosynthesis

B16F10 melanoma cells were seeded at a density of 2×10^4 cells/well in a 96-well plate and treated with 0.05–1.0 mg/mL of arbutin or the callus solution or extract. After 72 h incubation, the cells were washed twice with PBS (pH 7.4) and then dissolved with 150 μL of 1 N NaOH solution. Samples were incubated at 65 $^{\circ}\text{C}$ for 45 min and mixed to dissolve the melanin. The absorbance of the supernatant at 405 nm was then measured. The extent of melanin synthesis

in each sample was calculated by reference to the total melanin level in the untreated control over the experimental period.

Cell proliferation assay

To evaluate the effects of *Pyrus pyrifolia* var. *culta* callus extract on fibroblast proliferating ability, human fibroblast (CCD-986sk) cells were seeded at 5×10^3 cells/well in 100 μL of Dulbecco's modified Eagle's medium (DMEM) with 10 % (v/v) fetal bovine serum (FBS) and 1 % (w/v) penicillin/streptomycin, and cultured at 37 $^{\circ}\text{C}$ for 24 h. The serum concentration was then lowered to 0.05 % (v/v) and the cells were incubated for an additional 24 h. The cells were then treated with rhTGF- β or *Pyrus pyrifolia* var. *culta* callus solution or extract in DMEM containing 0.5% (v/v) FBS. After 24 h of incubation, 5 mg/mL WST-1 in PBS (pH 7.4; Roche Diagnostics, Mannheim, Germany) were added to each well followed by incubation at 37 $^{\circ}\text{C}$ for 2 h. The absorbance at 450 nm was measured, and the percentage cell viability was calculated relative to that of the untreated cells.

Procollagen synthesis assay

To assess whether the callus extract had anti-wrinkle activity (promotion of collagen synthesis), the level of procollagen type I C-peptide synthesized by fibroblasts was used as a marker of procollagen secretion. CCD-986sk cells (1×10^4 cells/well) in 100 μL of DMEM with 10 % (v/v) FBS were cultured in a 96-well plate. When 80 % confluence was attained, the medium was changed to DMEM supplemented with 0.5 % (v/v) FBS, and the cells were further cultured overnight. The cells were stimulated with various concentrations of rhTGF- β or the callus solution or extract in serum-free medium. After 24 h of incubation, the culture supernatants were collected, and procollagen type I C-peptide levels were determined using an enzyme-linked immunosorbent assay (ELISA) kit (Takara Bio, Shiga, Japan) according to the manufacturer's protocol.

In vitro scratch wound recovery assay

To examine the skin regenerative capacities of *Pyrus pyrifolia* var. *culta* callus extract, CCD-986sk cells were seeded (1×10^4 cells/well) in a 96-well plate and cultured in complete medium at 37 $^{\circ}\text{C}$ for 48 h to form confluent monolayers. After creation of cell-free zones (scratches) using a wound-maker, each well was washed twice with PBS (pH 7.4). The cells were then cultured at 37 $^{\circ}\text{C}$ with 500 ng/mL of rhTGF- β or 5 mg/mL of the callus solution or extract in serum-free

medium. Cell migration was monitored every 12 h by phase contrast imaging using an Incucyte Zoom microscope (Essen Bioscience Inc., Ann Arbor, MI, USA). The built-in image analysis software was used to detect cell edges and to generate overlay masks used to calculate cell recovery areas.

Statistics

All data are presented as mean \pm standard deviation (SD). Statistical analyses were performed by the Student's t-test between two groups or one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test among more than three groups. $P < 0.05$ was considered to be statistically significant.

RESULTS

Characteristics of callus extract of *Pyrus pyrifolia* var. *culta*

Pyrus pyrifolia var. *culta* callus was successfully induced after transferring the leaf explants into MS medium supplemented with 2.0 mg/L of picloram, used as a growth regulator. Moreover, the callus was friable and light yellow in color when cultured on MS liquid medium supplemented with 2.0 mg/L picloram; this was

not the case when other plant growth regulators such as BA and 2,4-D were used.

The total phenol contents of *Pyrus pyrifolia* var. *culta* callus solution and extract were 65.50 ± 1.13 and 50.80 ± 1.46 mg/L, respectively. The total contents of flavonoids in the callus solution and extracts were 15.90 ± 1.13 and 6.21 ± 1.76 mg/mL, respectively.

Figure 1 presents the DPPH radical scavenging activities of *Pyrus pyrifolia* var. *culta* callus solution and extract. Ascorbic acid, a positive control, exhibited radical scavenging activity. The callus extract also exhibited dose-dependent activity and a 78.7 % free radical-scavenging effect, which was similar to that of 500 μ M ascorbic acid (82.1 %). However, the callus solution exhibited marginal DPPH scavenging activity at a concentration <5 mg/mL and exhibited only 46.8 % free radical scavenging activity at 10 mg/mL.

Inhibition against tyrosinase and melanin biosynthesis

We measured the tyrosinase inhibitory activities of the callus solution and extract to evaluate the effects of the extract on melanogenesis.

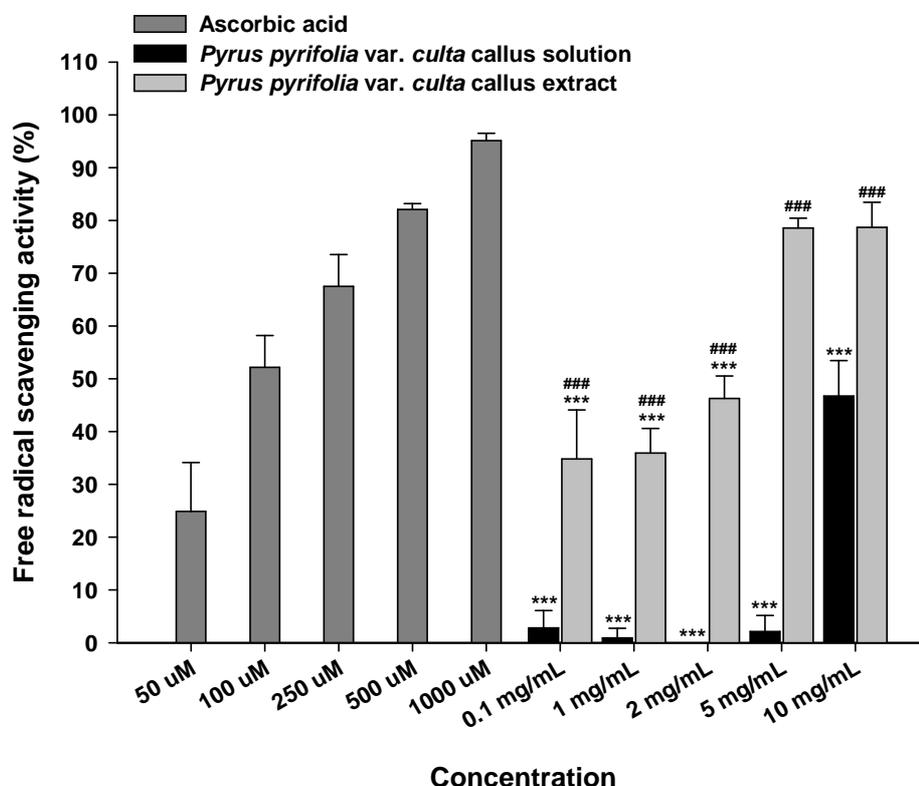


Figure 1: DPPH free radical scavenging activity of *Pyrus pyrifolia* var. *culta* callus solution and extract. Each value represents mean \pm standard deviation ($n = 4$ for each group); *** $p < 0.001$ compared with 500 μ M ascorbic acid; ### $p < 0.001$ compared with *Pyrus pyrifolia* var. *culta* callus solution

No significant reduction in tyrosinase activity or L-DOPA auto-oxidation was obtained with the callus solution at any concentration tested, while the extract marginally inhibited tyrosinase activity at concentrations greater than 10 mg/mL (Figure 2A). The extract inhibited L-DOPA oxidation in a dose-dependent manner; 10 mg/mL extract solution inhibited L-DOPA auto-oxidation by 13.6 ± 2.05 % compared with the untreated control (Figure 2B).

B16F10 cells. The *Pyrus pyrifolia* var. *culta* callus solution exhibited a significantly greater inhibitory effect on melanin production than did the arbutin or callus extract; melanin production in melanocytes treated with 0.1 mg/mL callus solution was inhibited by 51.40 ± 5.57 % compared with the untreated control. Arbutin and callus extract exhibited dose-dependent reductions in melanin content. The callus extract exhibited 44.60 ± 3.65 % inhibition of melanin synthesis at 1 mg/mL, 1.4-fold greater than arbutin at the same concentration.

Figure 3 presents the effects of *Pyrus pyrifolia* var. *culta* callus extract on melanin production in

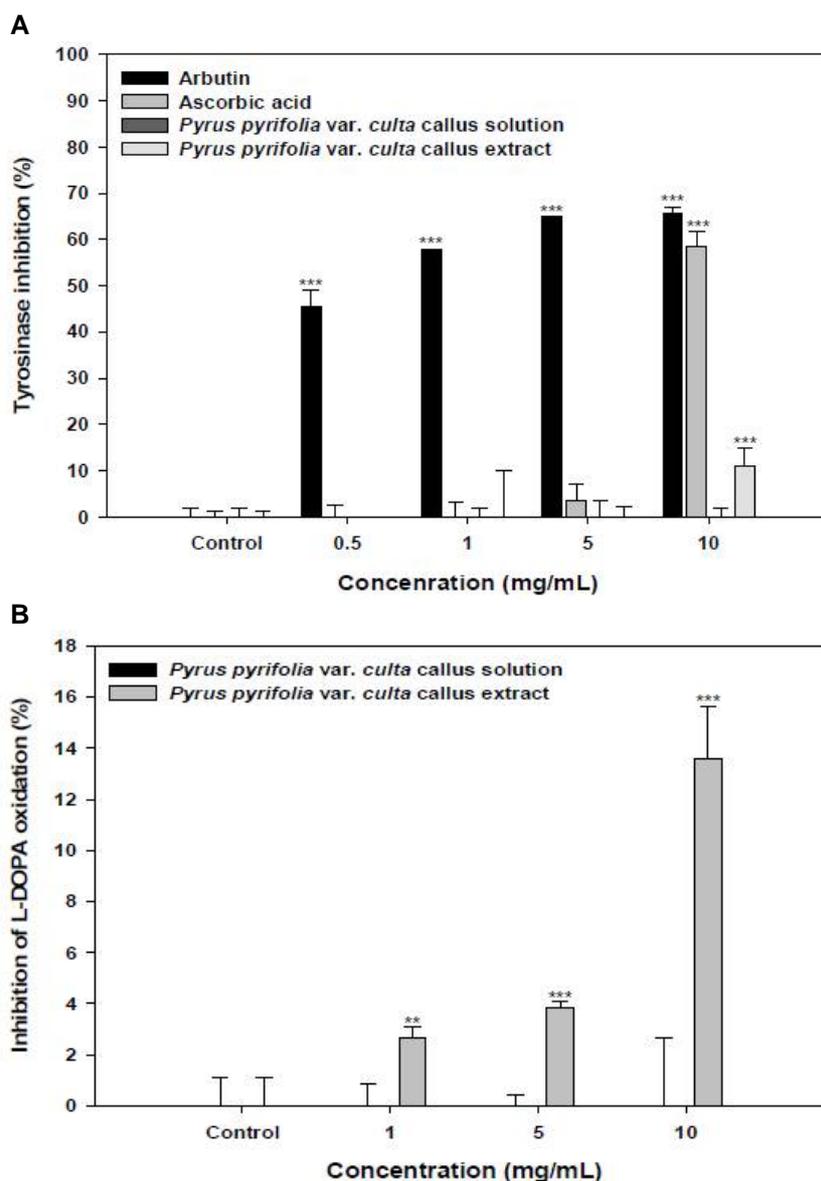


Figure 2: Effect of *Pyrus pyrifolia* var. *culta* callus solution or extract on mushroom tyrosinase activity. The relative activity of mushroom tyrosinase assayed on (A) L-tyrosine hydroxylation and (B) 3,4-dihydroxyphenylalanine (L-DOPA) oxidation in the absence or presence of different concentrations of each extract. Results are expressed as percentages of the control and each value represents the mean \pm standard deviation ($n = 4$ for each group); $p < 0.01$, $***p < 0.001$ compared with the untreated control

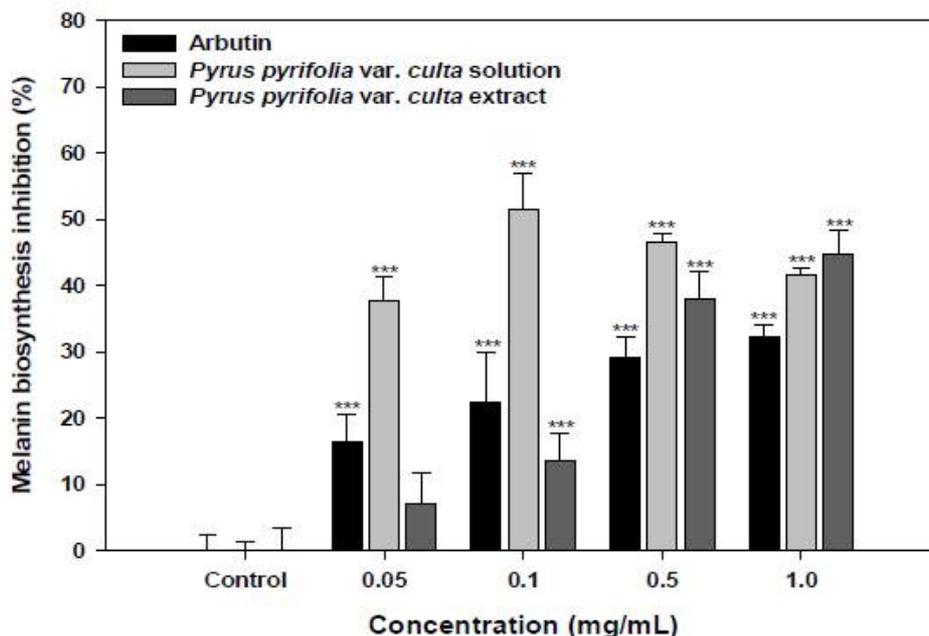


Figure 3: Inhibitory effect of *Pyrus pyrifolia* var. *culta* callus solution or extract on the melanin content of B16F10 melanoma cells. Results are expressed as percentages of the control and each value represents the mean \pm standard deviation (n = 4 for each group); ***p < 0.001 compared with the untreated control

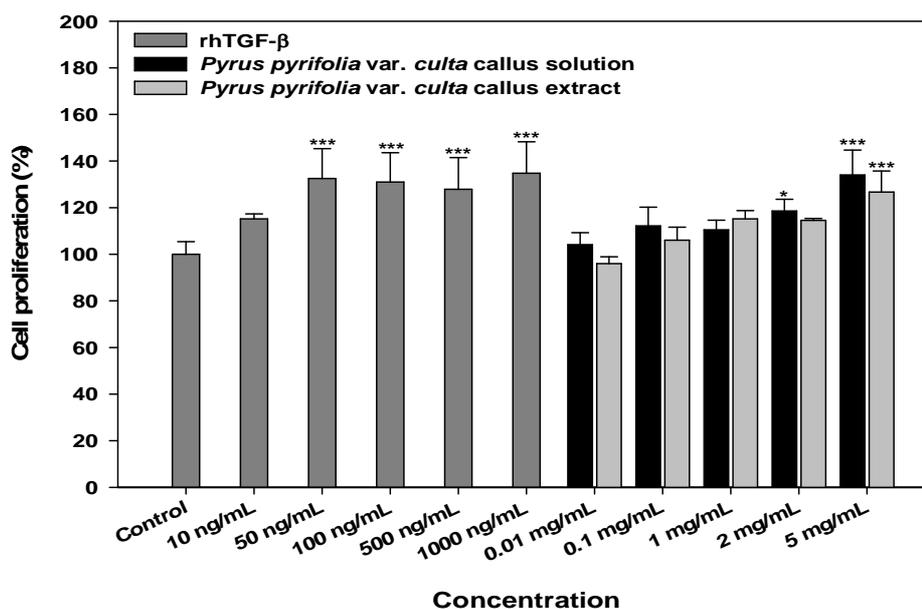


Figure 4: Relative proliferation of CCD-986sk cells after incubation with rhTGF- β , *Pyrus pyrifolia* var. *culta* callus solution or extract for 24 h. Each value represents the mean \pm standard deviation (n = 6); *p < 0.05, ***p < 0.001 compared with the untreated control

Cell proliferation

To evaluate the skin regenerating activity of *Pyrus pyrifolia* var. *culta* callus extract, we examined the effects of callus solution or extract on the proliferation of CCD-986sk cells. As shown in Figure 4, human fibroblasts proliferated significantly after treatment with rhTGF- β (positive control); the extent of proliferation in the presence of 50 ng/mL rhTGF- β was 133 \pm 12.8

% greater than that of the control group. The proliferation of fibroblasts was not increased significantly at concentrations <5 mg/mL of callus solution or extract. However, fibroblasts treated with 5 mg/mL of callus solution and extract exhibited 134 \pm 10.7 % and 127 \pm 9.00 % greater proliferation than the control group, respectively. Furthermore, the callus solution or extract did not cause any severe inhibition of cell viability and exhibited >100 % cell viability at all

concentrations tested. Thus, the *Pyrus pyrifolia* var. *culta* callus solution and extract were apparently well tolerated and appear not to have toxic effects on the skin.

Procollagen levels

Next, we investigated the effects of callus extract on collagen synthesis by human fibroblasts. As shown in Figure 5, the biosynthesis of procollagen type I C-peptide was increased significantly compared with the control group after the cells were treated with 10 – 1000 ng/mL rhTGF- β . Procollagen type I C-peptide production after treatment with 1, 2 and 5 mg/mL of callus solution was 2.13-, 2.01, and 2.07-fold higher than that of the control, respectively. Similarly, the *Pyrus pyrifolia* var. *culta* callus extract stimulated procollagen synthesis in a

dose-dependent manner; procollagen type I C-peptide synthesis was 2.04-fold greater than that of the control in fibroblasts treated with 5 mg/mL callus extract.

In vitro scratch wound recovery

In the *in vitro* scratch wound recovery assay, treatment with *Pyrus pyrifolia* var. *culta* callus solution or extract significantly accelerated wound closure compared with the control (Figure 6A). The recovery rates after 72 h of treatment with 5 mg/mL of the callus solution and extract were 134 and 172 % higher than those of the serum-free medium control group, respectively. The callus extract (5 mg/mL) exhibited rates accelerated by 128 and 106 % compared with the callus solution (5 mg/mL) and rhTGF- β (500 ng/mL), respectively (Figure 6B).

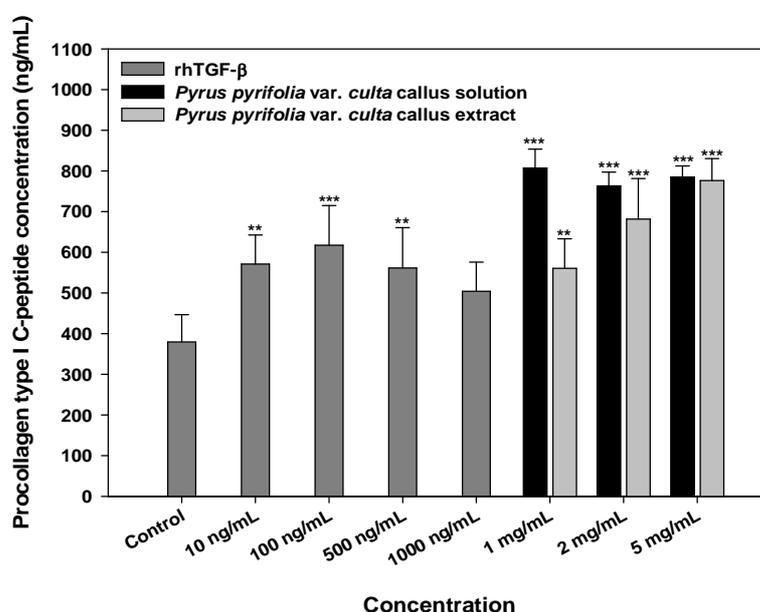


Figure 5: Procollagen type I C-peptide synthesis by CCD-986sk cells after incubation with rhTGF- β , *Pyrus pyrifolia* var. *culta* callus solution or extract for 24 h. Each value represents mean \pm standard deviation (n = 6); *p < 0.01, ***p < 0.001 compared with the untreated control

DISCUSSION

Many studies have reported that continuous exposure to ROS accelerates skin aging by compromising the antioxidant system, triggering melanogenesis and wrinkle formation [17,18]. Thus, use of fibroblasts and keratinocytes to protect against oxidative stress is an important strategy in skin anti-aging applications. In the present study, the *Pyrus pyrifolia* callus extracts were prepared from plant cell and tissue cultures, which have been suggested to be useful for *de novo* production of secondary metabolites including phenolics and flavonoids. Next, we measured the free radical-scavenging activity of the *Pyrus pyrifolia* callus extract using the DPPH

assay. As total phenolic and flavonoid levels were strongly correlated with anti-oxidant activity, these compounds may be responsible for the anti-oxidant properties of *Pyrus pyrifolia* calli.

To evaluate the effects of the *Pyrus pyrifolia* var. *culta* extract on melanogenesis, we measured the effects of the extract on tyrosinase activity; compounds with anti-oxidant activities usually exert whitening effects [19]. Tyrosinase, expressed by all organisms, is a multifunctional copper-containing enzyme that catalyzes two important reactions of melanin biosynthesis: hydroxylation of L-tyrosine to L-DOPA and oxidation of L-DOPA to dopaquinone [20,21]. In this study, the extract did not exhibit inhibition of

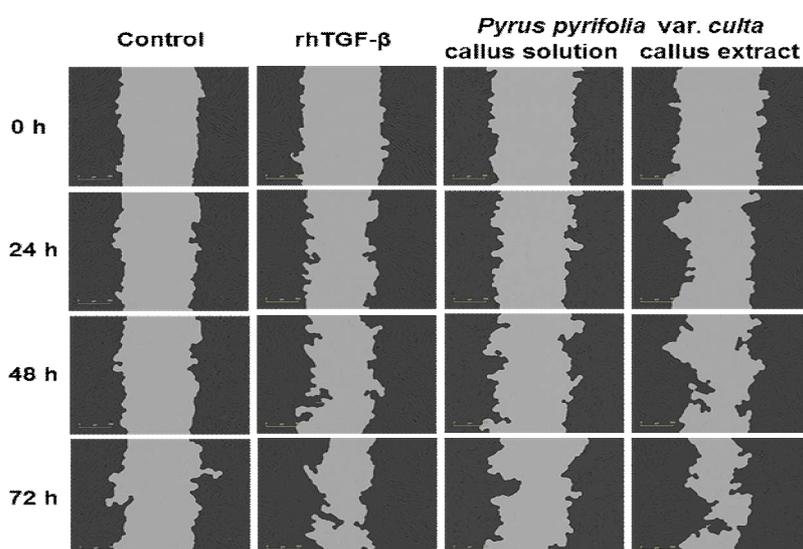
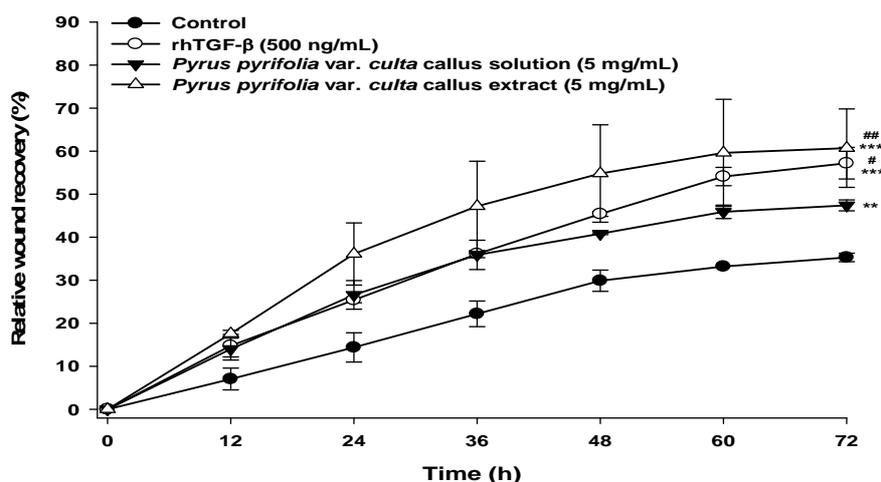


Figure 6: Relative scratch wound recovery of CCD-986sk cells after incubation with rhTGF- β , *Pyrus pyrifolia* var. *culta* callus solution or extract for 72 h. (top image) Time-course of relative scratch wound recovery area of CCD-986sk cells. (bottom image) Representative microscopic images of scratch wounds treated with rhTGF- β , *Pyrus pyrifolia* var. *culta* callus solution or extract. Each value represents mean \pm standard deviation ($n = 6$); * $p < 0.01$, ** $p < 0.001$ compared with the untreated control; # $p < 0.05$, ### $p < 0.01$ compared with *Pyrus pyrifolia* var. *culta* callus solution. Scale bar = 300 μm

tyrosinase activity at the concentrations tested, while it exhibited significant inhibition of DOPA auto-oxidation in melanogenesis. These results suggest that the anti-oxidant effects of the extract may play a significant role in decreased melanin synthesis [22].

When developing skin anti-aging ingredients for cosmetic applications, one strategy is to find materials that upregulate the collagen content in the dermis. Collagen, a fibrous protein, is a major component of the extracellular matrix (ECM) in the dermis that imparts tensile strength to the human skin [23]. Synthesis of collagen from its precursor procollagen occurs within ECM fibroblasts [24]. After hydroxylation of the proline and lysine residues of procollagen, the protein is

secreted into the ECM and transformed into collagen fibers via a series of polymerizations [25]. In this regard, *Pyrus pyrifolia* var. *culta* callus extract significantly enhanced the proliferation of fibroblasts, which has a stimulatory effect on collagen production.

The extracts of *Pyrus pyrifolia* var. *culta* have been also reported to inhibit glycation. Protein glycation is the term given to the accumulation of non-enzymatic products from reactions of proteins with glucose and other reducing sugars, triggering structural and functional modifications of tissue proteins and oxidative damage to tissues [26,27]. Recently, it has been shown that glycation creates stable active sites catalyzing free radical formation, accelerating normal aging

and contributing to the pathogenesis of age-related diseases [28]. Furthermore, inhibitory activity against glycation is known to correlate significantly with the anti-oxidative potency of the extracts [29]. Therefore, *Pyrus pyrifolia* var. *culta* callus extract may have broad and beneficial effects in skin anti-aging, which may be related to its glycation inhibitory and anti-oxidant activities.

CONCLUSION

The findings of this study indicate that *Pyrus pyrifolia* var. *culta* callus extract has higher total contents of phenolics than flavonoids, and that 10 mg/mL of the extract exhibits 78.7 % DPPH free radical-scavenging effect, similar to that of 500 μ M ascorbic acid. The extract has no anti-tyrosinase activity but L-DOPA oxidation coupled with melanin biosynthesis is inhibited significantly after treatment with 10 mg/mL of the extract. Cell proliferation and procollagen synthesis in fibroblasts are promoted by treatment with the extract. Moreover, the callus extract has a capacity to accelerate scratch wound. Therefore, *Pyrus pyrifolia* var. *culta* callus extract has potentials as an anti-aging and skin-lightening agent.

DECLARATIONS

Acknowledgement

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Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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