Effects of *Tiarella polyphylla* D. Don Callus Extract on Photoaging in Human Foreskin Fibroblasts Hs68 Cells

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Abstract

Ultraviolet (UV)-irradiation causes an overproduction of matrix metalloproteinases (MMPs) and collagen (COL) degradation causing skin sunburn, inflammation, or photoaging in human. In this study, we prepared callus from *Tiarella polyphylla* D. Don (*T. polyphylla*) stem and its phytochemical profiles were analyzed using HPLC-MWD. The effects of *T. polyphylla* callus extract evaluated against UVB-induced damage in human foreskin fibroblast (Hs68). Hs68 was exposed to UVB in the presence or absence of *T. polyphylla* callus extract at concentrations of 100 and 250 µg/mL. Cell damage caused by UVB was inhibited by *T. polyphylla* callus extract, which was tested by cell viability and caspase 3 activity in Hs68 cells. Further experiment revealed that *T. polyphylla* extract suppressed the level of MMP-1, but increased the level of type I procollagen. In addition, *T. polyphylla* callus extract inhibited UVB-mediated COL (-1 and -3) protein degradation and MMP (-1, 2, and -3) overexpression in Hs68. These results suggest that *T. polyphylla* callus extract has considerable potential as a cosmetic ingredient with anti-aging effects.

Keywords

*Tiarella polyphylla* D. Don, plant cell culture, photodamage, type I procollagen, MMPs

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that is in charge of its strength and resilience. However, when the skin is exposed to UV radiation, the collagen content decreases and is accompanied by the aberrant accumulation of glycosaminoglycans and elastin fibers. Matrix metalloproteinases (MMPs) are matrix-degrading enzymes that play important roles in photoaging as well as in diverse tissue degradation or remodeling. UV irradiation causes loss of collagen content through the stimulation of collagen breakdown and inhibition of procollagen biosynthesis. This process is mediated by the expression of MMPs which is responsible for the deterioration of ECM proteins.

**Tiarella polyphylla** D. Don (*T. polyphylla*) is an annual herb belonging to Saxifragaceae family and is distributed in Ulleung Island in Korea, as well as in China and Japan. It has been used for the treatment of asthma, audition trouble and skin eruptions. Several studies have analyzed the compounds of *T. polyphylla* callus extracts. Shen et al., identified the flavonoids including myricetin, astragalin, quercitrin, and myricitrin in *T. polyphylla*. Studies also reported that flavonoids possess several biological effects, including antiaging effect in human dermal fibroblasts. Park et al., isolated oleanolic saponins and tiarellic acid from the plant, which showed an anti-complementary effect. Moon et al., also reported that compounds from the *T. polyphylla* have protective effects on the UV-induced type I procollagen reduction in human dermal fibroblasts.

Despite of many reports on useful pharmacological properties of *T. polyphylla*, there have been no reports of plant tissue culture studies on *T. polyphylla* yet. Moreover, no studies have been considered the use of *T. polyphylla* callus to improve the skin photoaging. Here we reported the preparation of callus from *T. polyphylla* and characterize the phytochemicals by HPLC-MWD. Next, we evaluated the anti-aging efficacy of the callus extract in human foreskin fibroblast using a UVB-induced damage model.

### Results

**In Vitro Proliferation of Adventitious Shoots of T. Polyphylla**

The white calluses were successfully induced from stem explants of *T. polyphylla* when they cultured on 1/2MS1B03D medium after 4 weeks of incubation. These calluses were transferred to fresh culture medium and sub-cultured at 4 week-intervals (Figure 1). After subculture, proliferated calluses were collected carefully and freeze-dried for the assay of cell viability and antiaging effect.

**Phytochemical Profiles in T. Polyphylla Callus**

The phytochemical profiles in *T. polyphylla* callus were identified using HPLC-MWD. Referring to the previously reported compounds in *T. polyphylla*, 6 flavonoids were used as standard for analysis of the compositional profiles (Figure 2(A) and (B)). By chromatographic screening, 4 compounds including nicotiflorin (kaempferol 3-O-rutinoside), astragalin (kaempferol 3-O-glucoside), quercitrin (quercetin 3-O-rhamnoside), and myricitrin (myricetin 3-O-rhamnoside) were identified in *T. polyphylla*. Calluses (Figure 2(C)). The content of identified component in *T. polyphylla* callus extract was analyzed as 18.05 ± 0.04 ng/mg nicotiflorin (kaempferol 3-O-rutinoside), 6.62 ± 0.03 ng/mg astragalin (kaempferol 3-O-glucoside), 5.45 ± 0.03 ng/mg quercitrin (quercetin 3-O-rhamnoside), and 15.80 ± 0.06 ng/mg myricetin (myricetin 3-O-rhamnoside). On the other hand, the major

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**Figure 1.** Callus induction and proliferation of calluses from stem explants of *T. polyphylla*. (A) Four weeks old white calluses of *T. polyphylla*. (B) Enlarged view of stem derived white callus. Scale bars represent 1 cm (A) and 1 mm (B).
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components of the *T. polyphylla* callus extract could not be identified, and also the components with unclear resolution and retention time referred to standard components could not be assigned. Further studies should be carried out to isolate, identify, and characterize the main compounds in *T. polyphylla* callus extract.

**Cytotoxicity of *T. Polyphylla* Callus Extract in Hs68 Cells**

First, Hs68 cells were treated with various concentrations of *T. polyphylla* callus extract (100, 250, and 500 µg/mL) to investigate whether the callus extract has cytotoxic effect for 24 hours. The cytotoxicity was measured by MTT assay. As shown in Figure 3, *T. polyphylla* callus extract has no cytotoxic effect for human fibroblast up to 250 µg/mL. However, the cell viability was decreased at 500 µg/mL of callus extract, and even though the cytotoxicity was not statistically significant, further studies were conducted by testing callus extracts below 250 µg/mL.

**Effects of *T. Polyphylla* Callus Extract on UVB-Induced Damage in Hs68 Cells.** Next, the inhibitory effect of the extract on
UVB-induced damage in Hs68 cells was evaluated. Hs68 cells were incubated with different concentrations (100 and 250 µg/mL) of callus extract for 1 hours, and then exposed with UVB (100 mJ/cm²). After 24 hours, UVB exposure significantly decreased the viability of Hs68 cells compared to non-irradiated Blank group (Figure 4(A)). In contrast, the viability of Hs68 cells was increased significantly by *T. polyphylla* callus extract treatment (*P* < 0.001, Figure 4(A)). In addition, we tested the effects of *T. polyphylla* callus extract on UVB-induced apoptosis in Hs68 cells using caspase 3 activity assay.

**Figure 3.** Effects of *T. polyphylla* callus (PC4307) extract on the cytotoxicity in normal human foreskin fibroblast (Hs68). Cells were treated with various doses of callus extract for 24 hours. Cytotoxicity was determined with MTT assay. Values are means ± SEM of 3 independent experiments.

**Figure 4.** Effects of *T. polyphylla* callus (PC4307) extract on UVB-induced cell viability and caspase 3 activation in Hs68 cells. The cells were pretreated with *T. polyphylla* callus ethanol extract for 1 hours and irradiated with UVB (100 mJ/cm²) in Hs68 cells. After 24 hours, the viability and caspase 3 activity were analyzed using MTT assay and caspase 3 assay kit. (A) UVB-induced cell viability. (B) Caspase 3 activity. **P** < 0.001 vs Blank (without UVB irradiation); ***P** < 0.001 vs Control (CON, UVB irradiated group). Values are means ± SEM of 3 independent experiments.
UVB irradiation caused an increase in caspase 3 activity in Hs68 cells. This increase in caspase 3 was remarkably inhibited by *T. polyphylla* callus extract treatment at 100, 250, and 500 µg/mL (*P* < 0.001, Figure 4(B)), consistent with the increased cell viability. N-acetyl cysteine (NAC) was used as a positive control. The effects of callus extract were similar to that of NAC (1 µM, final concentration) treatment group.

**Regulation of UVB-Induced Type I Procollagen Degradation and Matrix Metalloproteinases (MMP)-1 Secretion by T. Polyphylla Callus Extract in Hs68 Cells.** To assess the inhibitory effects of *T. polyphylla* callus extract on type I procollagen degradation and MMP-1 production in UVB-exposed Hs68 cells, cells were exposed to UVB in presence or absence of *T. polyphylla* callus extract. After 24 hours, the secreted level of type I procollagen and MMP-1 was measured by ELISA. As shown in Figure 5(A), *T. polyphylla* callus extract substantially enhanced cellular levels of type I procollagen reduction by UVB (*P* < 0.05, *P* < 0.01). Also, MMP-1 levels were enhanced by 4-fold due to UVB irradiation compared to non-irradiated cells. Treatment with *T. polyphylla* callus ethanol extract restored MMP-1 levels (*P* < 0.001) (Figure 5(B)).

**Inhibitory Effect T. Polyphylla Callus Extract on COLs Degradation and MMPs Expression in Hs68 Cells.** Based on the above results, we expected that *T. polyphylla* callus extract might regulate COL (-1 and -3) and MMP (-1, -2, and -3) protein expression in Hs68 cells. UVB irradiation resulted in COL (-1 and -3) downregulation and MMP (-1, 2, and -3) overexpression in Hs68 cells (Figure 6). However, pretreatment of cells with callus extract prior to UVB irradiation restored COL (-1 and -3) degradation and MMP (-1, 2, and -3) overexpression. *T. polyphylla* callus extract at 250 µg/mL exhibited more potent effect than that of a positive control (NAC) on COLs degradation (Figure 6(A)) as well as MMPs expression (Figure 6(B)).

**Discussion**

*T. polyphylla* is the only species of Saxifragaceae family to be found in Korea, and grown naturally in Ullung island. The whole *T. polyphylla* is traditionally used for medical purposes, including inflammation and asthma. Previous studies have reported that the triterpene compound, tiarellic acid (3, 23-dihydroxy-20(29)-lupen-27- oic acid), plays an important role in inducing type I procollagen and the regulation of MMP (matrix metalloproteases)-1 protein expression. Shen et al., also reported that flavonoids from *T. polyphylla* exhibited a potent anti-complementary activities.

Plants are known as effective natural resources for treatment of oxidative stress, inflammation, and a variety of skin disorders. However, the provision of fresh materials, regardless of the season and the plant reproductive cycle have been a limitation to their application in the pharmaceutical and cosmeceutical products. The plant tissue and cell culture techniques were used for overcoming barriers and for uniformly controlled production of plant-derived bioactive components.

The awareness of cosmetic products of botanical origin on skin has increased in the most recent years among consumers. Because of people generally believe that such products are safety, this practice continues to trend upwards.

The functional properties of the skin contain both a number of fibroblasts, which are responsible for the modulation of extracellular matrix (ECM) components, and the integrity of collagen in the dermis. Chronic ultraviolet (UV) irradiation causes remodeling of the skin and induces photoaging...
including wrinkling, pigmentation, and less elasticity of the skin.\textsuperscript{24,25} Globally, environmental problem have reduced ozone in the stratosphere, resulting in ambient UVB. UVB radiation increases risk of damage in skin, including photoaging and photocarcinogenesis.\textsuperscript{26} Jung et al., reported that UVB (100 mJ/cm\textsuperscript{2}) increased MMP-1 secretion and downregulated type I procollagen protein in Hs68 cells.\textsuperscript{27}

In the present study, the \textit{T. polyphylla} callus were prepared from plant cell cultures. Next, we evaluated whether \textit{T. polyphylla} callus extract can be used to regulate UVB induced photaging in human foreskin fibroblast.

UVB exposure induced phototoxicity in human dermal fibroblasts, which was characterized by a decrease of cell viability as well as cell death.\textsuperscript{28} As presented in Figure 4, \textit{T. polyphylla} callus extract protected its toxicity by increasing cell viability and inhibition of caspase 3 activation against UVB irradiation. The callus extract has no cytotoxicity in an effective dose range (Figure 3).

Type I collagen is the most abundant protein in skin connective tissue which is composed of other ECM proteins. Type I procollagen, a soluble precursor of procollagen, is secreted from fibroblast and proteolytically processed to produce from insoluble collagen fibers.\textsuperscript{29} Fisher et al. have reported that type I and III collagen are reduced chronically photodamaged human skin.\textsuperscript{11} UV irradiation leads to degradation of ECM proteins, including type I collagen, and induces the MMPs expression.\textsuperscript{14,30} Earlier studies have been reported that MMPs activation is a major cause of the ECM protein degradation, which induces photoaging.\textsuperscript{11,14}

We found that \textit{T. polyphylla} callus extract regulated the type I procollagen reduction and MMP-1 (collagenase-1) secretion in human dermal fibroblasts by UVB irradiation (Figure 5).

According to previous studies, MMP-1 and MMP-2 (gelatinase-A) are regulated to COL-1 and -2 cleavage.\textsuperscript{31} Generally, MMP-1 is mainly responsible for biomarker of photoaging, and promoted skin aging, thus leading to wrinkle formation and initiated collagen degradation. MMP-2 was leading to reduction of collagen content by \textit{ex vivo} stress.\textsuperscript{24} It has been reported that MMP-3 induces proMMP-1 activation and continuously the secretion of the pro-MMP to form other MMPs.\textsuperscript{30}

Flavonoids are well known to exhibit inhibitory and anti-aging effects on UV-induced MMP activation in human dermal fibroblast. In particular, flavonoids, including kaempferol, quercetin and myricetin, have been reported to have a significant inhibitory effect on skin aging caused by UV irradiation. Mechanistically, the hydroxyl groups of flavonoid aromatic rings and collagenase can induce enzyme inactivation.\textsuperscript{32} It has been also reported that hydrophobic interactions between the flavonoid aromatic rings and collagenase can induce enzyme inactivation.\textsuperscript{32,33} Kanashiro et al., reported that flavonoids such as myricetin, kaempferol, and quercetin significantly inhibited elastase activity.\textsuperscript{34}

The present study revealed that \textit{T. polyphylla} callus extract significantly normalized the reduction of type I procollagen content and increase the level of MMP-1 in Hs68. In addition, \textit{T. polyphylla} callus extract inhibited UVB-mediated COL-1 and -3 protein degradation as well as MMP-1,-2 and -3 protein
expressions in Hs68 (Figure 6). We assigned that flavonol compounds, which are nicotiflorin, astragalin, quercitrin, and myricitrin, in *T. polyphylla* callus through HPLC profile, and it is assumed that these flavonol compounds delayed aging in Hs68 cells caused by UVB. However, the main compounds have not been identified and should be clarified through further research.

This study evidences that *T. polyphylla* callus can prevent photaging in human skin fibroblast by inhibiting MMP expression and its therapeutic and cosmetic applications remain to be explored.

### Materials and Methods

#### Chemicals and Antibodies

Chemicals and cell culture materials were obtained from following sources: All chemicals used were of analytical grade. High glucose Dulbecco's modified Eagle's medium (DMEM), Phosphate-buffered saline (PBS), penicillin and streptomycin, and fetal bovine serum (FBS) were obtained from Invitrogen (Carlsbad, CA, USA). The reference chemicals were obtained from Cell Signaling Technology Inc. (Beverly, MA, USA). The reference chemicals were obtained from Sigma-Aldrich.

#### Induction and Proliferation of Callus Derived From Stem of *T. Polyphylla*

Whole plants of *T. polyphylla* were collected from Ulleung island in Korea. Whole plants were sterilized in 70% (v/v) EtOH for 0.5 minutes, soaked in 0.8% sodium hypochlorite (NaOCl) solution for 20 minutes. After surface sterilization, plants were rinsed thoroughly with sterile distilled water. To remove the remaining NaOCl solution, these washing processes were repeated 3 times. After washing, remaining moisture was removed by sterilized filter paper (Advantec, 70 mm). The plants were dissected into 3 segments; leaf, petiole and stem. To induce callus, stem explants (approximately 5 mm in length) were transferred to 1/2MS 35 basal medium supplemented with 0.4 mg/L thiamine-HCl, 600 mg/L myo-inositol, 1 mg/L 6-benzylaminopurine, 0.3 mg/L 2,4-dichlorophenoxycetic acid, 3% (w/v) sucrose, and 0.4% (w/v) Gelrite (1/2MS1B03D). The pH of medium was adjusted to 5.8 with 1 n NaOH. The cultures were maintained at 25 °C in the dark. Each treatment of 10 explants with 3 replicates. Otherwise mentioned, all cultures were maintained at 25 °C in the dark. The callus line (KCTC PC4307, BP1421782) of *T. polyphylla* established in this study was deposited into Korean Collection for Type Cultures (KCTC).

### Preparation of *T. Polyphylla* Extract

After 4 weeks of incubation, rapidly growing white calluses from stem explant of *T. polyphylla* were transferred to fresh medium and further incubated in the dark at 25 °C. The calluses of *T. polyphylla* were sub-cultured at 4-week-interval. After 4 weeks of incubation, rapidly growing white calluses were collected carefully. These calluses were freeze-dried, and ground into a fine powder. The grinded callus powder (50 mg) was treated with ethanol (3.5 ml) and sonicated several times at room temperature for 2 days to produce an extract. The solvent was evaporated under N2 gas atmosphere and dried extract was stored at -80 °C for further experiments.

### Analysis of Phytochemicals

The *T. polyphylla* calluses were solubilized at 10 mg/mL in methanol and filtered through a 0.22 µm disposable PTFE filter membrane (Thermo Scientific, USA). The phytochemical of *T. polyphylla* calluses were analyzed by liquid chromatography (Agilent Technologies 1200 series, Santa Calara, CA, USA) coupled with multiwavelength detector (254 nm) by using a Aegispak-L C18 (4.6 × 200 mm, 3 µm) column at 35 °C. The flow rate was maintained at 0.55 mL/minutes. The mobile phase A consisted of 0.1% aqueous formic acid while mobile phase B was acetonitrile (HPLC grade, Thermo Scientific, USA). The gradient was performed as follows: 7 minutes 10% B, 11 minutes 25% B, 17 minutes 35% B, 21-24 minutes 45% B, 30-32 minutes 55% B, 43-49 minutes 70% B, 50 minutes 10% B. The compounds were identified according to their retention time and UV spectra by comparison with those obtained using standard compounds.

### Cell Viability Assay

Cell viability was analyzed as described following our previous method with some modifications. Briefly, human fibroblasts (Hs68 cells, ATCC, Manassas, VA, USA) were plated 96-well culture plates (1 × 10⁵ cells/well) and incubated for 24 hours with cellus extract and then exposed to UVB (100 mJ/cm²) using UV Crosslinker (Analytik Jena AG, Jena, Germany). The distance of irradiation of UV light to the sample was 15 cm and the time of exposure was 25 s/100 mJ/cm². After irradiation, cells were further incubated in the presence or absence of Callus extract for 24 hours and cell viability was estimated using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay.

### Measurement of Caspase-3 Activity

Caspase-3 activity was measured using the caspase-3 colorimetric assay kit (Abcam, Cambridge, UK), following the manufacturer’s instructions. This assay is based on the detection of the amount of DEVD-pNA substrate cleaved by cell lysates to release the chromophore of p-nitroaniline (p-NA). Briefly,
Hs68 cells were harvested and lysed using cell lysis buffer. Lysed cells were centrifuged at 10,000 × g, 4 °C for 10 minutes. Caspase-3 activity was assessed by measuring the absorbance at 400 nm using an ELISA reader (Multiskan Go, Thermo Scientific, Waltham, MA, USA).

Measurement of Type I Procollagen and MMP-1
To determine the expression level of Type I Procollagen and MMP-1 as described previously,12 Hs68 cells (5 × 10⁵ cells/well) were treated with various concentrations (100 ~ 500 µg/mL) of Callus extract for 24 hours. After treatment, cells were exposed to UVB (100 mJ/cm²) and further incubated for 24 hours. The culture medium was then harvested and the chemokine production levels in the supernatants were measured using ELISA kits according to the manufacturer’s instructions.

Immunoblotting
Protein extraction and immunoblotting of cells were performed as previously.12 Protein concentration in the cells was measured using a Bio-Rad protein assay kit with bovine serum albumin as a standard. Cell lysates containing equal amounts (20 µg per lane) of total protein were separated by electrophoresis on SDS-PAGE gel (8% or 10%) and then transferred to polyvinylidene difluoride (PVDF, GE Healthcare, Little Chalfont, Buckinghamshire, UK) membranes. The membrane was blocked with 5% skim milk in TBS-T and subsequently incubated with specific primary antibodies (1:2500) at 4 °C. After overnight incubation, the horseradish peroxidase-conjugated secondary antibodies (1:5000) incubated for 2 hours at room temperature, bands were visualized using an enhanced chemiluminescence (ECL) system (Bio-Rad, Munich, Germany). All immunoreactive bands were visualized by enhanced chemiluminescence detection system (Amersham imager 600, GE Healthcare, Buckinghamshire, UK).

Statistical Analysis
Statistical Analysis of the data was performed using Student’s t test was used to compare differences between 2 groups and one-way ANOVA followed by the Tukey post hoc test. Results are presented as means ± standard deviation (SD). The value P < 0.05 was considered significant for all experiments.

Declaration of Conflicting Interests
The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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