ORIGINAL ARTICLE

Enhancement of Human Hair Growth Using *Ecklonia cava* Polyphenols

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Background: *Ecklonia cava* is a brown alga that contains various compounds, including carotenoids, fucoidans, and phlorotannins. *E. cava* polyphenols (ECPs) are known to increase fibroblast survival. The human dermal papilla cell (hDPC) has the properties of mesenchymal-origin fibroblasts. **Objective:** This study aims to investigate the effect of ECPs on human hair growth promotion *in vitro* and *ex vivo*. **Methods:** MTT assays were conducted to examine the effect of ECPs on hDPC proliferation. Hair growth was measured using *ex-vivo* hair follicle cultures. Real-time polymerase chain reaction was performed to evaluate the mRNA expression of various growth factors in ECP-treated hDPCs. **Results:** Treatment with 10 μg/ml purified polyphenols from *E. cava* (PPE) enhanced the proliferation of hDPCs 30.3% more than in the negative control ($p < 0.001$). Furthermore, 0.1 μg/ml PPE extended the human hair shaft 30.8% longer than the negative control over 9 days ($p < 0.05$). Insulin-like growth factor-1 (IGF-1) mRNA expression increased 3.2-fold in hDPCs following treatment with 6 μg/ml PPE ($p < 0.05$). Vascular endothelial growth factor (VEGF) mRNA expression was also increased 2.0-fold by 3 μg/ml PPE ($p < 0.05$). Treatment with 10 μg/ml PPE reduced oxidative stress in hDPCs ($p < 0.05$). **Conclusion:** These results suggest that PPE could enhance human hair growth. This can be explained by hDPC proliferation coupled with increases in growth factors such as IGF-1 and VEGF. Reducing oxidative stress is also thought to help increase hDPCs. These favorable results suggest that PPE is a promising therapeutic candidate for hair loss. (**Ann Dermatol 28(1) 15~21, 2016**)

INTRODUCTION

Ecklonia cava, an edible marine brown alga, is produced abundantly in Korea and Japan. *E. cava* contains various bioactive compounds and derivatives including phlorotannins, peptides, carotenoids, and fucoidans[1]. It has been reported that phlorotannins and marine plant polyphenols from *E. cava* possess various biological functions including radical scavenging, antiplasmin inhibition, antimutagenic, bactericidal, and tyrosinase inhibiting activities[2]. The phlorofurofucoeckol A has been shown to have a protective effect against cellular toxicity[3]. In addition, eckol and dieckol, the major phlorotannins isolated from *E. cava*, increase fibroblast survival by reducing reactive oxygen species (ROS)[4].

Dermal papilla cells (DPCs) comprise a group of specialized fibroblasts. DPCs play a critical role in regulating hair follicle development and periodic regeneration[5,6]. Human hair growth has a unique repetitive cycle composed of the anagen, catagen, and telogen phases[7]. The hair cycle is completely influenced by DPCs; if the DPCs are in a pathological state, various hair loss disorders occur[8]. Topical minoxidil, a well-established therapeutic for

various types of alopecia, mainly affects DPCs[8,9]. Therefore, *E. cava* polyphenols (ECPs), which can increase fibroblast survival, might promote hair growth. Dieckol was recently reported to stimulate hair growth via DPC proliferation and/or 5α-reductase activity inhibition in rat vibrissa. ECPs were also shown to promote hair growth in C57BL/6 mice. However, they did not exhibit efficacy in human cells or follicles[10]. The effects of ECPs on human hair growth have not been thoroughly investigated. To the best of our knowledge, only one study to date has tested the effect of ECPs on hair growth in human cells. The results of that study showed that of the ECPs, dioxinodehydroeckol is responsible for the hair growth–promoting activity via overexpression of insulin-like growth factor-1 (IGF-1)[11]. Other ECP-isolated compounds may enhance human hair growth; however, the mechanism by which ECPs promote hair growth has yet to be determined.

In the present study, we investigated the effect of three ECPs, dieckol, phlorofurofucoeckol A, and purified polyphenols from *E. cava* (PPE), and purified polyphenols from *E. cava* enriched with eckol (PPEE) on human hair growth enhancement *in vitro* and *ex vivo*.

MATERIALS AND METHODS

Test materials

ECPs including dieckol, phlorofurofucoeckol A, and PPE as well as PPEE were supplied by Botamedi Inc. (Jeju, Korea) in dried powder form. PPE and PPEE were generated as described below. Dried *E. cava* was washed with copious amounts of water to remove salt and water-soluble components and then extracted with 95% ethanol. The resulting extract was separated and concentrated *in vacuo* to yield a dark-brown powder (PPE). PPE was further extracted using ethyl ether and the extract was concentrated in vacuo yielding a light brown powder (PPEE). Notable components of PPE and PPEE identified by high performance liquid chromatography (Waters, Milford, MA, USA) include dieckol (8.2% and 16.8%, respectively), phlorofurofucoeckol A (1.5% and 3.5%, respectively), and eckol (0.8% and 1.9%, respectively). The PPEE was further partitioned with organic solvents to yield dieckol and phlorofurofucoeckol A. Each substance was mixed with dimethyl sulfoxide (DMSO) and diluted as required for the experiments. DMSO also was used as a negative control.

Isolation of human hair follicles and DPC culturing

Prior to the study, the protocols were approved by the Seoul National University Hospital Institutional Review Board (H-1112-096-390). Twelve healthy male volunteers aged 20~50 years were recruited and occipital scalp tissue containing more than 100 hair follicles was obtained by excisional biopsy. Informed consent was obtained prior to the procedure. Anesthesia was administered by local injection of lidocaine and the wound was closed with a simple suture. Candlelight-shaped human dermal papillae (hDP) were obtained under stereomicroscope (Olympus, Tokyo, Japan) by cautious dissection of the obtained scalp tissue into single hair follicles, incising the follicle at the level just above the hDP using two 26-G needles and softly squeezing the hDP. Hair follicles morphologically considered to be in the anagen stage were used in this study. Dissociated hDP were incubated in Dulbecco's modified Eagle medium (DMEM; Welgene, Daegu, Korea) supplemented with 10% fetal bovine serum (FBS, Welgene), basic fibroblast growth factor (bFGF, 1 μl/100 ml), and antibiotics (100 mg/ml streptomycin and 100 U/ml penicillin) at 37°C in 5% CO_2. The culture medium was changed every other day. DPCs were harvested when they reached 80% confluence using 0.05% trypsin and then successively subcultured.

Thiazolyl blue [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (MTT) assay

To determine the effect of ECPs on hDPC survival and proliferation, viability was measured using the MTT assay[12]. Subsequent to counting cells numbers using a hemocytometer, hDPCs were seeded into 96-well plates at 1.0×10^4 cells/well. These cells were cultured for 24 hours and then the medium was changed to serum-free medium. Following another 24-hour culture period, the cells were treated with various concentrations of compounds including dieckol (1~100 μM), phlorofurofucoeckol A (1~100 μM), PPE (0.1~10 μg/ml), and PPEE (0.1~10 μg/ml) as well as DMSO as the negative control. Treatment with 0.1 μM minoxidil served as the positive control. Next, 20 μl of MTT solution (0.5 mg/ml) was added to each well and incubated for 4 hours at 37°C in the dark. Following supernatant removal, 200 μl of DMSO was added to dissolve the formazan crystals and then incubated for 20 minutes at room temperature. The samples were assayed by measuring the absorbance at 570 nm using an enzyme-linked immunosorbent assay reader.

Human hair follicle organ culture study

Isolated human scalp hair follicles from scalp tissue were cultured *ex vivo*. Scalp skin tissue samples containing more than 100 hair follicles were obtained from 12 healthy volunteers aged 20~50 years and dissected into single individual hair follicles. Isolated hair follicles were cut at the level of the sebaceous duct to generate follicles approximately 3.5 mm in length that were cultured in

Williams E medium (Gibco BRL, Gaithersburg, MD, USA) containing 10 ng/ml hydrocortisone, 10 μg/ml insulin, 2 mM L-glutamine, and 100 U/ml penicillin at 37°C in 5% CO_2 for 12 days. ECPs were added to this culture medium at final concentrations (dieckol and phlorofurofucoeckol A, 1~100 μM; PPE and PPEE, 0.1~10 μg/ml). DMSO, the vehicle, was used as the negative control, while 1.0 μM minoxidil served as a positive control. The culture medium was changed every third day and the shaft elongation of each hair follicle was measured directly using a stereomicroscope (Olympus).

Quantitative real-time polymerase chain reaction (RT-PCR)

Quantitative RT-PCR was performed to measure the mRNA expression of IGF-1, vascular endothelial growth factor (VEGF), transforming growth factors (TGF)-β1, and β-catenin. The hDPCs were treated with various concentrations of each substance (dieckol and phlorofurofucoeckol A, 10~100 μM; PPE and PPEE, 1~10 μg/ml). The negative control was DMSO. Following 24-hour culturing, total RNA was isolated from the hDPCs using RNA iso-Plus (Takara Bio Inc., Otsu, Japan) and chloroform sequentially then clarifying the supernatant by centrifugation at 13,000 rpm for 15 minutes at 4°C. Following treatment with DNase I (Roche Pharmaceuticals, Welwyn Garden City, UK) to remove the genomic DNA and the addition of isopropanol to this supernatant, RNA was precipitated by centrifugation at 13,000 rpm for 10 minutes. A First Strand cDNA Synthesis Kit (Fermentas, Sankt Leon-Rot, Germany) was used for cDNA synthesis reaction according to the manufacturer's instructions. To quantitatively estimate mRNA expression, PCR was performed on a 7500 RT-PCR System (Applied Biosystems, Foster City, CA, USA) using SYBR Premix Ex Taq (Takara Bio Inc.) according to the manufacturer's instructions. The PCR conditions were 95°C for 2 minutes followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. The data were analyzed using the $2^{-\Delta\Delta CT}$ method and presented as the fold-change in expression relative to the control; values were normalized to *36B4*, the control gene. The experiment was performed in triplicate and repeated with specimens from two to four individuals.

Measurement of ROS

To determine the presence of oxidative stress, ROS production was measured using the oxidation-sensitive probe dichlorodihydrofluorescein-diacetate (DCF-DA; Invitrogen, Carlsbad, CA, USA). The detected ROS were fluoresced by DCF-DA (20 μM), and fluorescence-activated cell sorting (FACS) cytometry was performed according to the manufacturer's instructions. The hDPCs were treated with H_2O_2. Following 2 hours incubation, hDPCs treated with only DMSO were considered the negative control. In the experimental groups, hDPCs were treated with various ECPs (100 μM dieckol, 100 μM phlorofurofucoeckol A, 10 μg/ml PPE, and 10 μg/ml PPEE) for 2 hours. The ROS scavenging effect of the ECPs was examined. The experiment was performed in triplicate and repeated at least three times.

Statistical analysis

The Mann-Whitney test was used for the MTT assay, quantitative RT-PCR, and ROS measurement. A Wilcoxon signed rank test was used to assess the results from *ex vivo* human hair follicle cultures. Results are presented as means$\pm$standard error of the mean in graphs. All quoted *p*-values are two-tailed and significance was defined as values < 0.05. The statistical analyses were done by using the IBM SPSS Statistics 21.0 software package (IBM Co., Armonk, NY, USA).

RESULTS

Enhanced proliferation of hDPCs

The MTT assay results show that all ECPs had a significant effect on hDPC proliferation. Particularly, 100 μM dieckol significantly enhanced hDPC proliferation (170.6$\pm$22.4%, Fig. 1A). Phlorofurofucoeckol A (1 μM and 100 μM) also demonstrated significant effects (131.3$\pm$11.9% at 1 μM; 208.8$\pm$49.6% at 100 μM, Fig. 1B). PPE was less efficient than dieckol and phlorofurofucoeckol A; however, the effects of 10 μg/ml PPE was significant compared to the negative control (130.3$\pm$19.6%, Fig. 1C). PPEE, which contains more dieckol and phlorofurofucoeckol A than PPE, also showed a 129.7$\pm$10.1% fold-change relative to the negative control (10 μg/ml, Fig. 1D).

Increased hair growth in *ex vivo* human hair follicle cultures

Ex vivo cultures of human hair follicles were obtained from 12 individual volunteers. Hair follicles treated with 0.1 μg/ml PPE grew 1.38$\pm$0.15 mm after 6 days and 1.74$\pm$0.18 mm after 9 days, which was significantly longer than the growth of hair treated with the negative control (1.09$\pm$0.18 mm after 6 days; 1.33$\pm$0.20 mm after 9 days). However, dieckol, phlorofurofucoeckol A, PPEE, and other PPE concentrations did not significantly enhance hair growth (in length) compared with the negative control (Fig. 2).
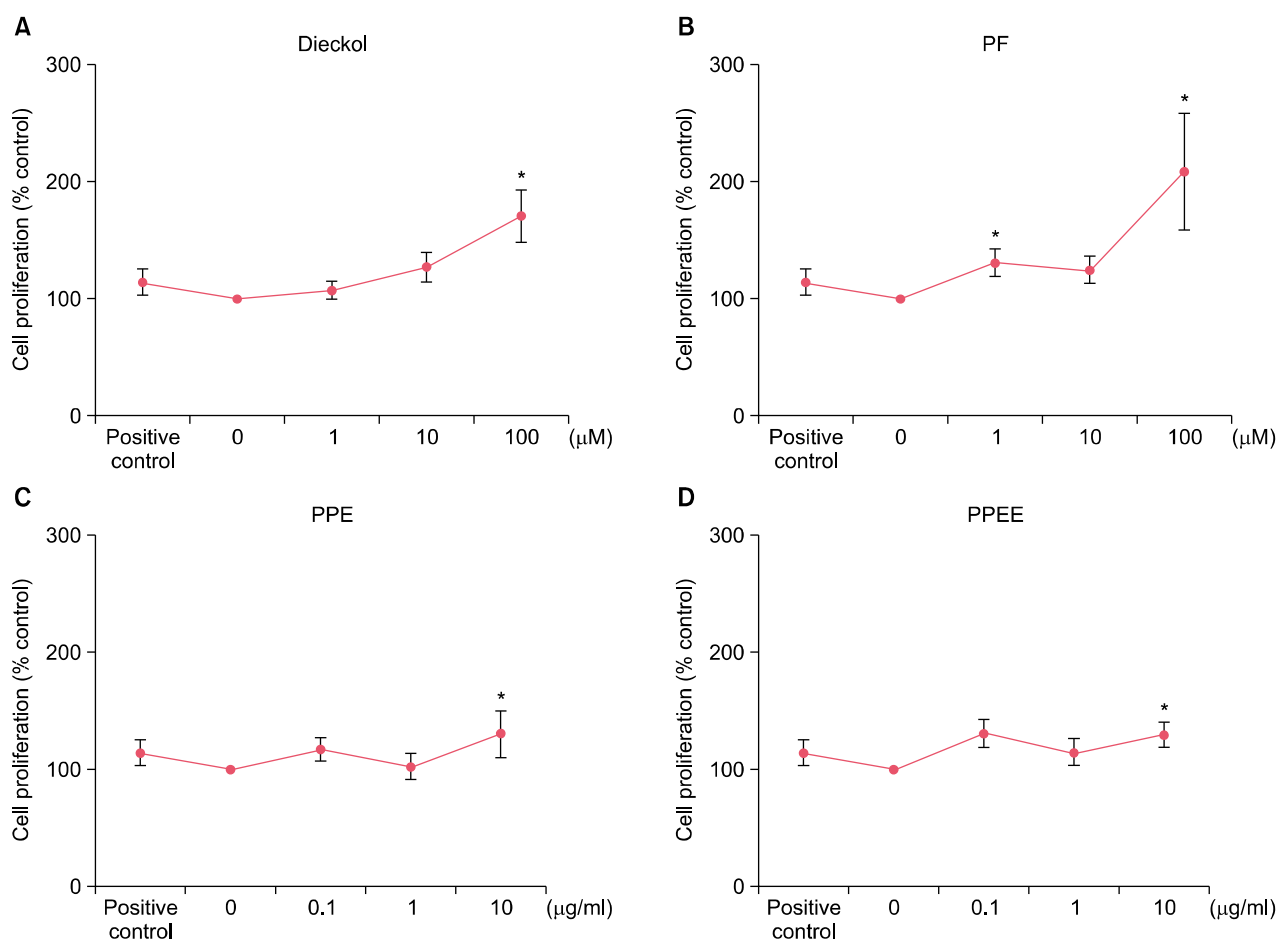
Fig. 1. Thiazolyl blue assay to test the effect of *Ecklonia cava* polyphenols on human dermal papilla cell proliferation. (A) Dieckol, (B) phlorofurofucoeckol A (PF), (C) purified polyphenols from *E. cava* (PPE), (D) purified polyphenols from *E. cava* enriched with eckols (PPEE). Positive control, minoxidil 0.1 μM. *$p<0.05$ compared with the negative control.

Increased IGF-1 and VEGF mRNA expression

The mRNA expression levels of IGF-1, VEGF, TGF-β1, and β-catenin in hDPCs were evaluated using quantitative RT-PCR. No significant differences were apparent in the mRNA levels of hDPCs treated with dieckol, phlorofurofucoeckol A, or PPEE, compared with the negative control. However, there were significant differences in the mRNA expression of IGF-1 in hDPCs treated with PPE compared with the negative control. IGF-1 mRNA expression was increased 3.2-fold in hDPCs following PPE (6 μg/ml) treatment (Fig. 3A). VEGF mRNA expression was also increased 2.0-fold by PPE (3 μg/ml) treatment ($p<0.05$, Fig. 3B). However, the mRNA expression of TGF-β1 and β-catenin in hDPCs was not significantly influenced by PPE (data not shown).

Antioxidant effect of ECPs in hDPCs

Dieckol, phlorofurofucoeckol A, PPE, and PPEE significantly reduced ROS levels in hDPCs. The ROS level measured by FACS cytometry was 86.0% in dieckol-treated, 77.5% in phlorofurofucoeckol A-treated, 88.8% in PPE-treated, and 90.7% in PPEE-treated hDPCs relative to the level in vehicle-treated hDPCs. ECPs scavenged ROS and reduced oxidative stress in hDPCs ($p<0.05$, Fig. 4).

DISCUSSION

DPCs play an essential role in controlling hair growth[5]. Factors affecting DPC function are crucial for identifying novel hair growth enhancers. Therefore, in this study we focused on DPC to determine the efficacy and mechanism of hair growth enhancement using *E. cava*.

All ECPs including dieckol, phlorofurofucoeckol A, PPE, and PPEE, exhibited good effects on hDPC proliferation. Dieckol has been reported to multiply immortalized rat vibrissa DPCs[10]; we confirmed that dieckol promotes the proliferation of DPCs in humans as well. A previous study demonstrated that the ethyl acetate–soluble fraction of *E. cava* increased hDPC proliferation 130.6% at 0.01 μg/ml
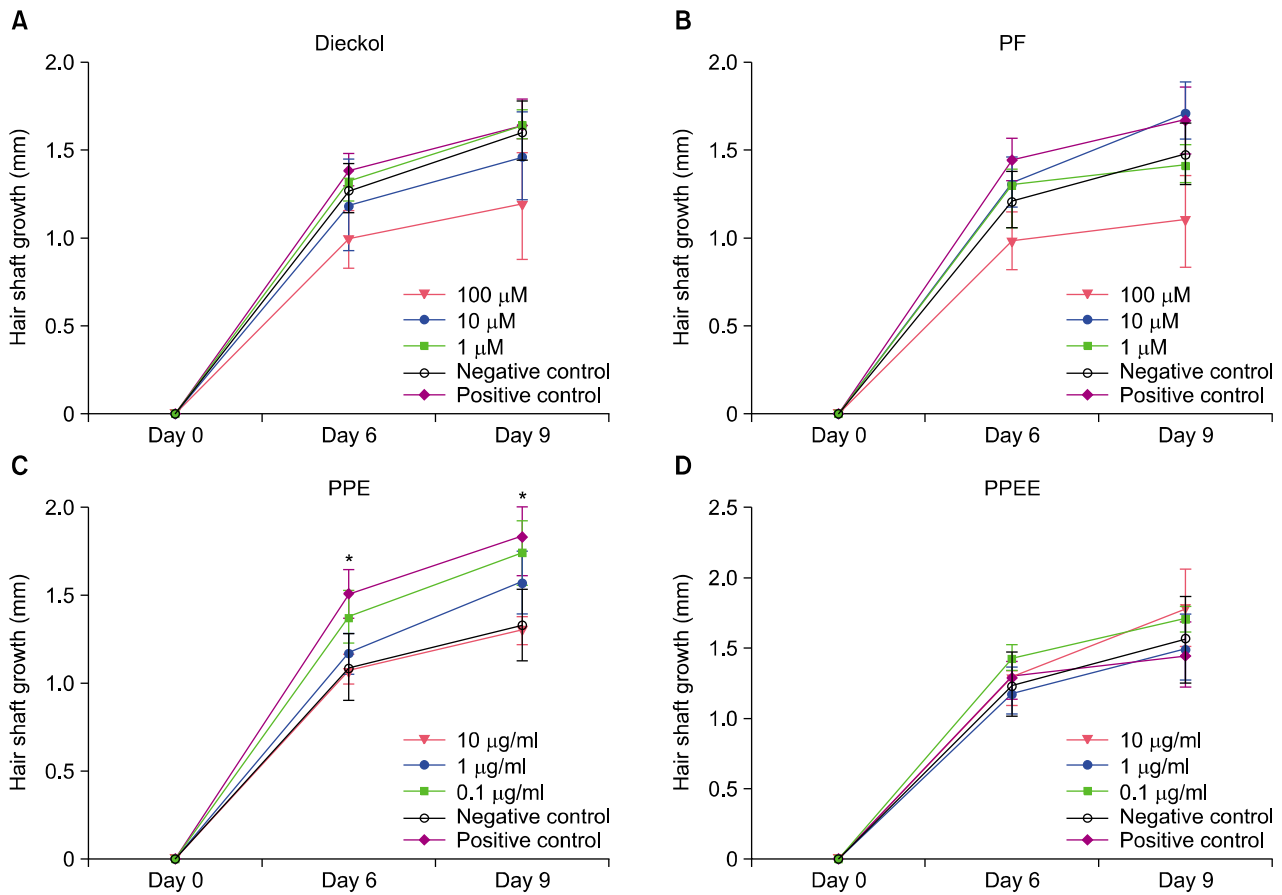
Fig. 2. Time-course of hair growth (length) over 6 and 9 days. (A) Dieckol, (B) phlorofurofucoeckol A (PF), (C) purified polyphenols from *Ecklonia cava* (PPE), (D) purified polyphenols from *E. cava* enriched with eckols (PPEE). Positive control, minoxidil 1.0 μM. *$p<0.05$ compared with the negative control.
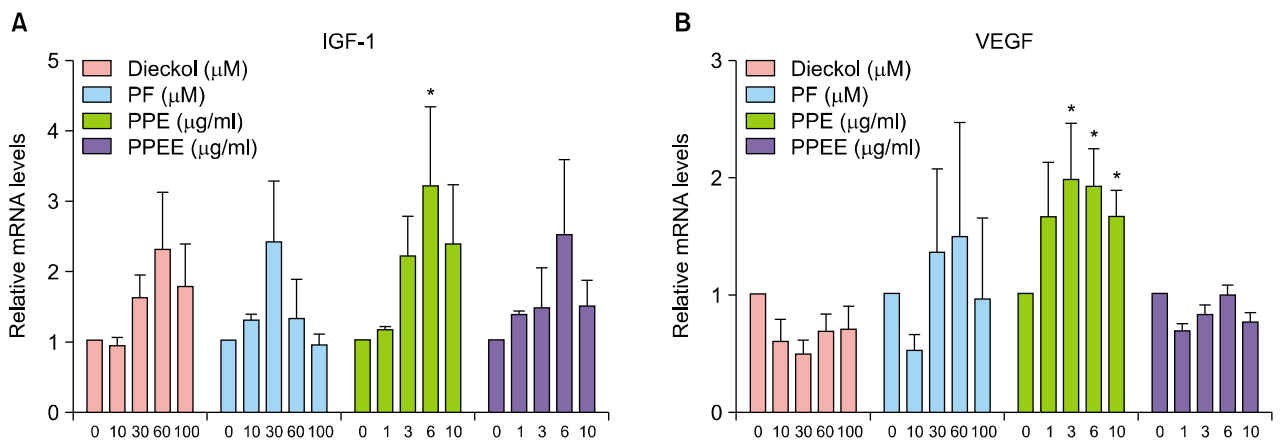

Fig. 3. Quantitative real-time polymerase chain reaction for (A) IGF-1 and (B) VEGF in PPE-treated hDPCs. IGF-1: insulin-like growth factor-1, VEGF: vascular endothelial growth factor, hDPCs: human dermal papilla cells, PF: phlorofurofucoeckol A, PPE: purified polyphenols from *Ecklonia cava*, PPEE: purified polyphenols from *E. cava* enriched with eckols. *$p<0.05$, compared with the negative control.

and 138.6% at 0.1 μg/ml compared with the control[11]. These results are similar to our PPE data (130.3% at 10 μg/ml). In addition, we found that dieckol and phlorofurofucoeckol A, which are single phlorotannins, are present in PPE. These compounds are more effective than PPE for hDPC proliferation (170.6% at 100 μM dieckol and 208.8% at 100 μM phlorofurofucoeckol A). A high dose of PPE (70 μg/ml) did not increase hDPC proliferation
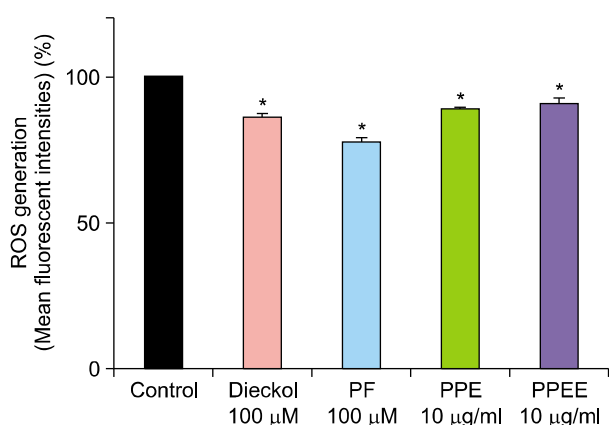
Fig. 4. Oxidative stress in hDPCs was detected using DCF-DA and FACS was performed to measure the antioxidant effect of *E. cava* polyphenols. hDPCs: human dermal papilla cells, FACS: fluorescence-activated cell sorting, ROS: reactive oxygen species, PF: phlorofurofucoeckol A, PPE: purified polyphenols from *Ecklonia cava*, PPEE: purified polyphenols from *E. cava* enriched with eckols. *$p < 0.05$ compared with the negative control.

(99.1%; data not shown). Based on these results, we hypothesized that there is an optimal ECP concentration range for enhancing hDPC proliferation and that dieckol and phlorofurofucoeckol A may be important ECP compounds for hair growth promotion. Therefore, we predicted that PPEE, which contains highly concentrated dieckol and phlorofurofucoeckol A, would promote hair growth more efficiently than ordinary PPE.

However, in *ex vivo* human hair follicle cultures, dieckol, phlorofurofucoeckol A, and PPEE failed to show significant hair shaft elongation results, while low concentrations of PPE (0.1 μg/ml) resulted in significant elongation. Higher concentrations of ECPs did not guarantee a superior effect on hair shaft elongation. Since natural plant extracts usually exhibit a certain level of toxicity in cells, ECPs are thought to have a narrow optimal concentration range for cell proliferation. The ECP effect seems to vary depending on cell type and test conditions such as *in vivo*, *ex vivo*, or *in vitro*.

These *ex vivo* human hair follicle culture results were supported by the RT-PCR findings. Increased mRNA expression of IGF-1 and VEGF was observed in PPE-treated hDPCs. Many peptides are associated with hair growth, including IGF-1, VEGF, TGF-β1, and β-catenin[13,14]. IGF-1 is a basic, 70 amino acid peptide that promotes cell growth, survival, and differentiation[15]. IGF-1 plays a critical role in regulating cellular proliferation and migration during hair follicle development[16]. IGF-1 expression in the DPC has been shown to correlate with the therapeutic efficacy of androgenic alopecia treatment[17]. VEGF is a homo-dimeric, heparin-binding glycoprotein that plays im-

portant roles in mediating angiogenesis during development[18]. Follicle-derived VEGF promotes perifollicular vascularization, hair growth rates, and increased follicle and hair thickness[19]. TGF-β1, the growth factor that regulates cell growth, apoptosis, and differentiation, is involved in the regulation of hair follicle regression by inducing apoptosis and inhibiting keratinocyte proliferation[20,21]. β-catenin is essential for the formation of hair placodes during embryogenesis and is required for the differentiation of skin stem cells in adults. When β-catenin is deleted subsequent to hair follicle formation, hair is completely lost following the first hair cycle[22]. In this study, PPE significantly influenced IGF-1 and VEGF but not TGF-β1 or β-catenin.

Androgen, considered to be the major etiology of androgenic alopecia, increases ROS in hDPCs with overexpressed androgen receptors. Androgen-inducible ROS enhances TGF-β1 secretion from hDPCs. Interestingly androgen-induced TGF-β1 secretion was reversed by a ROS scavenger[23], indicating that antioxidants can promote hair growth. The well-established antioxidant, vitamin C, stimulates DPC growth and promotes hair shaft elongation *in vitro* and *in vivo* in animal experiments[24]. The potent antioxidant, green tea epigallocatechin-3-gallate, is also reported to enhance hair growth[25]. ECPs are another group of well-known antioxidants. We confirmed that ROS produced by hDPCs are significantly scavenged by ECPs. This result suggests that ECPs possess hair growth promoting effects *via* reducing ROS.

In this study, we did not determine why dieckol and phlorofurofucoeckol A did not show significant results in *ex vivo* human hair follicle cultures and the reason for the discrepancy between the RT-PCR results and the significant results of the MTT assay. PPEE did not exhibit superior efficacy to that of ordinary PPE in *ex vivo* cultures or RT-PCR in contrast to our predictions. Although all of the ECPs showed a good proliferative effect on hDPCs, PPE, a mixed and crude phlorotannin compound, is thought to be the key substance that affects hair growth. It is possible that other molecules have major effects on hair growth. PPEE contains more dieckol and phlorofurofucoeckol A than ordinary PPE, whereas other crude phlorotannin compounds are removed from PPEE through the extraction process. A certain combination of dieckol, phlorofurofucoeckol A, and other ECPs might prove more effective than a single molecule since dieckol and phlorofurofucoeckol A are very easily oxidized to inactive forms. Other unknown molecules within PPE may be necessary to maintain the stability of those molecules. It has been reported that a mixture of several antioxidants is more stable and synergistic than single antioxidants[26]. Further studies

are required to identify the most effective specific or mixed compounds.

In summary, our results suggest that PPE could promote human hair growth via the proliferation of hDPCs with ROS scavenging and increasing growth factors such as IGF-1 and VEGF. PPE constitutes a promising alternative therapeutic choice for various hair loss disorders.

ACKNOWLEDGMENT

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