Antioxidant Effect of Extracellular Vesicles Derived From Houttuynia Cordata

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Abstract

Background: Edible plants have been used as effective ingredients in the pharmaceutical and cosmetic industries, but studies on extracellular vesicles (EVs) derived from these plants have been limited. In partucular, *Houttuynia cordata* has been used as a traditional remedy to treat various diseases and to relieve skin problems because it contains antioxidant, antibacterial and anti-inflammatory properties.

Methods: We isolated EVs from *Houttuynia cordata,* and measured size distribution and surface charge. In addition, we performed the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay to measure the antioxidant effect.

Results: The physical properties of EVs from *Houttuynia cordata* reflect their nanoscale dimensions and intrinsic properties of EVs. Similar to mammalian exosomes, the size of EVs derived from this plant also ranged from 30 to 200 nm. Additionally, the viability of HFDPCs at a concentration of 500 μg/mL of EVs was greater than 85%. It showed a strong antioxidant activity more than 80%, which was 1.5 times higher than that of the extract by the general method.

Conclusion: EVs of *Houttuynia cordata* with antioxidant effect is expected to be used in various industrial applications including cosmetics, food, and pharmaceuticals.

Keywords: edible plants, Houttuynia cordata, extracellular vesicles, antioxidant effect

Introduction.

Extracellular vesicles (EVs) play an important role not only intercellular, but also interspecies communication by delivering proteins, and RNAs [1, 2]. Recent studies on edible plants-derived EVs have verified safety of EVs to humans, and even efficacy involved in human health and disease [3-5]. There is a report that Raimondo *et al.* showed anticancer activities from EVs of Citrus limon (lemon), and Mu *et al.* have also studied that EVs from edible plants induce activation of intestinal immune cell homeostasis [6, 7].

We previously confirmed the anti-melanogenic effects from leaves of *D. morbifera* [8], and anti-metastatic and anti-cancer cell effects from sap of *D. morbifera* [9, 10]. In this study, we focused on extracellular vesicles derived from *H. cordata*. *H. cordata* is called heartleaf and traditionally people of these regions were using this plant extract for folk medisines [11]. It is known that *H. cordata* extract have antioxidant effect against oxidative stress, anti-inflammatory, anti-bacterial, anti-viral [12, 13].

Oxidative stress may be an important mechanism contributing to hair loss [14]. Park *et al.* have demonstrated that antioxidants can prevent oxidative stress [15], and *H. cordata* has been found to exhibit strong antioxidant properties [11]. We hypothesized that EVs from *H. cordata* would effectively promote hair regeneration and prevent hair loss. Here, we investigated the effects of EVs derived from *H. cordata.* We found that leaf-derived extracellular vesicles extracted from *Houttuynia cordata* have properties similar to those of mammalian exosomes. Our study assessed the antioxidant between EVs and extracts from *H. cordata* using DPPH radical scavenging, and H-EVs showed inhibitory activity against DPPH radicals compared to the extract. Our results showed that H-EVs promoted human follicular dermal papillary cells (HFDPs) migration. Collectively, these findings suggest that natural *H. cordata*-derived EVs can be used ingredient for the treatment of hair loss in hair care cosmetics.

Materials and Methods.

2.1. Isolation of Houttuynia cordata leaf-derived extracellular vesicles (H-EVs)

We collected fresh leaves of *Houttuynia cordata* from Daegu-si, South Korea. Unlike the general method for isolating plant vesicles, we have developed a method for isolating EVs from *Houttuynia cordata* leaf to facilitate industrial application. *Houttuynia cordata*-derived

EVs were isolated by processing the leaves with a mixer grinder plus extractor, passing the resulting crude leaf extract through filter paper, and centrifuging the obtained extract at 3,000× g for 20 min. Then, large debris was removed by filtering the supernatant through a membrane step by step from1 um to 0.22 μm to remove and then the filtered EVs were concentrated by centrifuging the sample at 5000× g for 10 min at 4 ℃ in an Amicon Ultra-4 PL 100 K concentrator (Merck Millipore, Darmstadt, Germany).

2.2. Size characterization of isolated H-EVs

Hydrodynamic size distribution was determined by measuring intensity of light scattered from H-EVs using dynamic light scattering (DLS). Collected H-EVs were measured in a thermostatic cell at 20 ℃. For zeta potential measurement, the H-EVs were diluted with distilled water at a ratio of 95:3 (v/v), and inserted into a folded capillary cell (DTS1070; Malvern Instruments). The diluted samples were detected using a Zetasizer nano ZS90 system (Malvern Panalytical, Malvern, UK).

2.3. Protein quantification

The total protein contents associated with H-EVs was assessed using a Pierce BCA assay kit for bicinchoninic acid. A standard curve was prepared by pipetting 10 μL of BCA standard solution and 200 μL of BCA working reagent into each well, and immediately incubating the mixture at 37 ℃ for 30 min. Absorbance at 562 nm was evaluated with a microplate reader (BioTek, Winooski, VT, USA).

2.4. 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay

The H-EVs were diluted to various concentrations (0.01, 0.05, 0.1, 0.2, 0.5, 1.0, 2.0, 4.0, 8.0 mg/mL, 50 μL), in ethanol. To a total volume of 200 μL, 0.2 mM DPPH ethanol solution and H-EVs for each concentration are mixed at 3:1 and reacted at room temperature for 30 min. Then, the absorbance values were determined at 515 nm using a microplate reader.

The scavenging activity was calculated using the following the equation [15]:

$$\text{DPPH scavenging activity (\%)} = [(A_{control} - A_{sample}) / A_{control}] \times 100$$

Where, $A_{control}$ is the absorbance of the control without H-EVs and A_{sample} is the absorbance of the tested sample. Ethanol was used as a blank. 0.2 mM DPPH solution was used as a negative control. The positive controls were L-ascorbic acid.

2.5. Cell culture and viability assays

Human follicular dermal papillary cells (HFDPs) were purchased from Cell Engineering for Origin (Seoul, Korea), and cultured in CEFOgro™ Human Dermal papilla growth medium (CEFO, Seoul, Korea). The cells were cultured in a humidified incubator with 5% CO_2 atmosphere at 37 ℃. For cell viability assays, HFDP cells were seeded 3 x 10^5 cells in a volume of 100 μL in 96-well plates. After incubating for 24 h, H-EVs at 1, 10, 50, 100, 200, 500 μg/mL were treated with 10 μL for 24h, respectively. Thereafter, 10 μL of EZ-cytox agent (Daeil Lab, Seoul, Korea) was added to each well and the plate was incubated for at least 1 h at 37 ℃. Then, the plate was then gently shaken before reading the absorbance at 450 nm using a microplate reader (BioTek, Winooski, VT, USA).

2.6. Wound healing assay

HFDPCs were seeded 3 × 10^5 cells/well in a volume of 70 μL using culture-inserts (ibidi, Germany), and placed in 24-well plates. Culture-inserts were removed carefully from 24-well plate, after incubation for 24h. Then, each well was treated with H-EVs at dose-dependent concentrations of 1, 10, 50, 100, 200, 500 μg/mL.

The widths of cell migration from the edge of monolayer were photographed using JuLI™ Br Bright-cell Movie Analyzer (NanoEntek, Korea). The images of wounded monolayer were taken at 0 and 24 h, and analyzed with Image J software (National Institutes of Health, Bethesda, MD, USA), and all experiment was repeated at three times.

The wound healing was calculated using the following the equation [16]:

$$\text{Wound healing} = [(A_{0h} - A_{24h}) / A_{0h}] \times 100$$

Where, A_{0h} is the area of the initial wound calculated after scratching, and A_{24h} is the area of the unhealed wound that remained at 24 h.

Ethanol was used as a blank. 0.2 mM DPPH solution was used as a negative control. The positive controls were L-ascorbic acid.

Results and Discussion

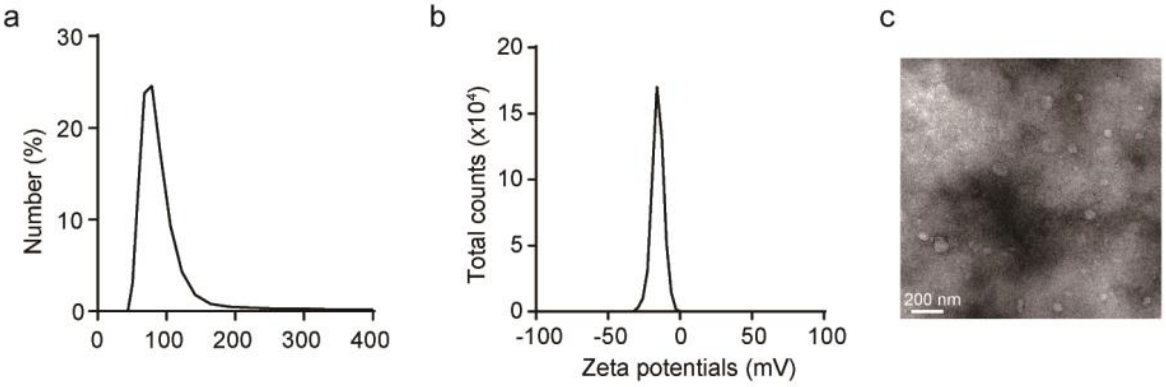

Figure 1. Characterization of *H. cordata*–derived extracellular vesicles (H-EVs). (a) Dynamic light scattering (DLS) measurements of particle size. (b) Zeta potential distribution of H-EVs. (c) TEM image of H-EVs.

1. Characterization of EVs from H. cordata

To investigate EVs from *H. cordata,* we isolated the extraction, and then centrifugation and filtration steps. Their size distribution was measured using dynamic light scattering (DLS), were approximately 100 nm (Figure 1a). We measured the zeta potentials of H-EVs, and their are surface charge, and related to electrophoretic mobility within charged colloidal dispersion. The observed zeta potential of H-EVs were approximately −20 mV. The high zeta potential result in stability of electrostatic mobility, and stable colloidal particles is known to have a zeta potential of ±20 mV. Thus, given their negative charge (−20 mV) of H-EVs exhibit stable mutual repulsion within suspension (Figure 1b). Transmission electron microscopy (TEM) images revealed that the EVs were nearly spherical vesicles in shape and had a size of approximately 100 nm, which is consistent with mammalian exosomes (30–200 nm).

Our analysis of size and properties of leaf-derived extracellular vesicles extracted from *Houttuynia cordata* showed that these EVs have physicochemical properties similar to those of mammalian vesicles. These plant-derived EVs offer the multiple benefits of small size, low toxicity, high uptake and envrironmental safety, could serve as next-generation therapeutic delivery systems for the treatment of other disease.

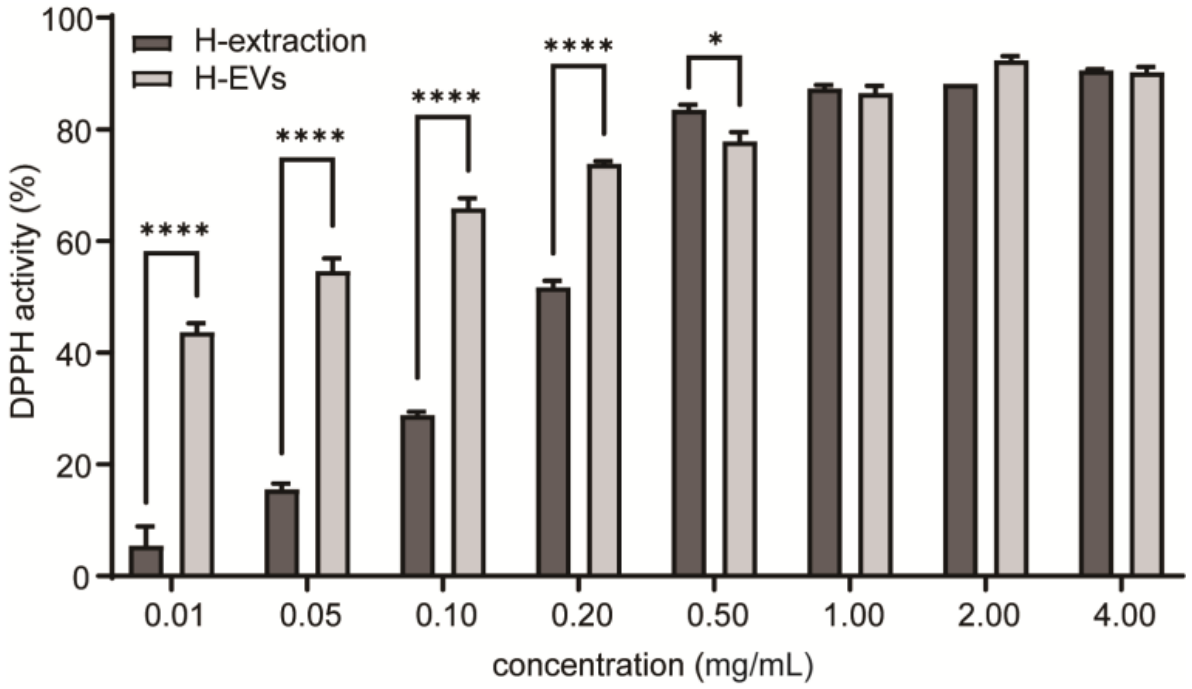

Figure 2. Determination of DPPH radical scavenging activity. Data are presented as means ± standard error of the mean (SEM) (*$p < 0.05$, *$p < 0.001$)

2. *Antioxidant activity using DPPH*

Naturally occuring plant antioxidants have the potential to reduce oxidative damage and suppress reactive oxygen species (ROS) through the inhibition of radical-producing enzymes and the enhancement of an antioxidant defense system. The conventional extract of *Houttuynia cordata* has been found to possess potent antioxidant properties. The DPPH assay provides one of the most widely used methods and rapid way for evaluating samples to scavenger free radical. This method determines the electron donation ability of samples resulting in the reduction of a purple DPPH radical to stable molecule. We compared to potential to reduce oxidative damage between extracellular vesicles and extraction from *Houttuynia cordata*. The results showed that H-EVs had superior scavenging activity to extracts *Houttuynia cordata* in DPPH assays. At a low concentration of 100-500 μg/mL, it was confirmed that the antioxidant effect was higher in H-EVs compared to the extracts. In particular, antioxidant of H-EVs was increased at greater than 37% compared to the extract

of that when the H-EVs concentrations was 100 μg/mL. From the concentration of 1 mg/mL, there was little difference between the H-EVs and extracts from *Houttuynia cordata*. Bioactive components in H-EVs exhibited their antioxidant activities in a dose-dependent manner. A recent study has been shown that phenolic compounds of rice bran extract have potent antioxidant activities to remove oxidizing free radicals, and high flavonoid contents of green tea extract induced hair follicles growth and inhibited apoptosis of dermal papilla cells [17].

Houttuynia cordata contains a wide range of polyphenols such as, rutin, quercetin, hyperoside, quercitrin, and chlorogenic acid, which have been considered to be responsible for the antioxidant activity [18]. The results may be explained as potent phenolic compounds of *Houttuynia cordata*, and its contents of EVs is expected to larger than that of extracts. Because EVs are separated by removing large debris through squential centrifuge and filtration, compared to the process of separating extracts. As a result, H-EVs showed superior antioxidant activity to extracts *Houttuynia cordata.*

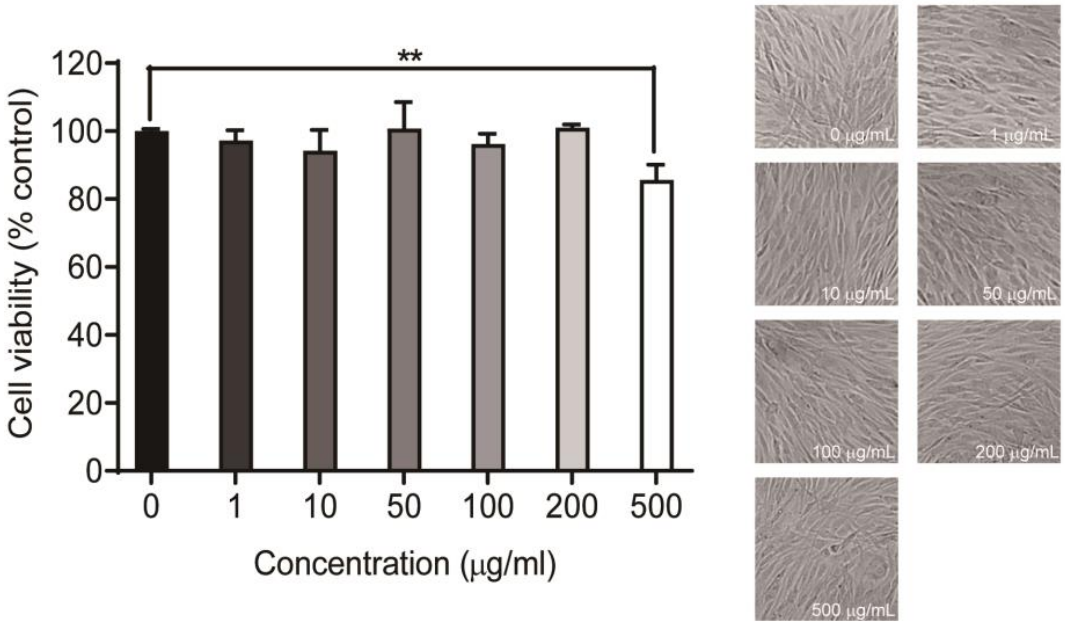

Figure 3. The percentage of cell viability on Human dermal papilla cells of EVs from *H. cordata*

3. *Proliferation effects of* EVs from *H. cordata* on Human dermal papilla cell.

To assess the cytotoxic effects of H-EVs, we treated human dermal papilla cells with 1, 10, 50, 100, 200, 500 μg/mL for 24 h and assessed their viability. As shown in Figure 3, cell viability was maintained nearly 100%, and the viability for 500 μg/mL of H-EVs was about 85%. No cytoxicity was observed up to a concentration of 200 μg/mL. To understand whether H-EVs could influence HFDP cell migration, *in vitro* model of wound scratch assay was performed in the absence or presence of 1, 10, 50, 100, 200, 500 μg/mL of H-EVs. We treated the cells with H-EVs and measured proliferation with imageJ assay after 24 h. We found that HFDP cell migration was generally enhanced by 24 h treatment with all H-EVs. In particular, 10 μg/mL H-EVs treatment significantly increased in HFDP cell migration rate compared to minoxidil, the positive control (Figure 4). We also found that the H-EVs treatment accelerated wound healing of the initial area than no treatment. HFDP cell proliferation increased more than 60 % treated with H-EVs at concentration 1, 10 μg/mL (Figure 4), which is possibility as an excellent natural new materials of hair growth. Wound healing process is complex and highly regulated for maintaining the skin barrier function. It was confirmed that the wound healing process appeared even at a low concentration of H-EVs that did not show toxicity. As naturally occurring plant-derived extracellular structures is nano-sized vesicles, it is important parameter for cellular processes, and may provide a new direction for future improvements in pharmaceutical or cosmeceutical applications.

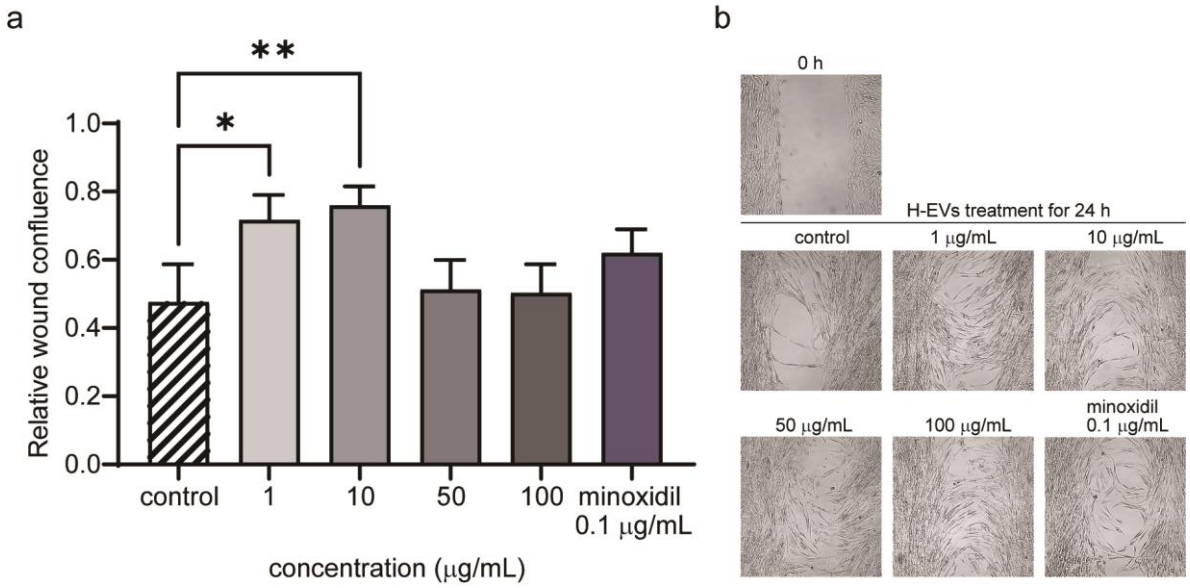

Figure 4. Migration of human dermal papilla cells treated with various concentrations of EVs from *H. cordata.* (a) summary data showing a comparison of migration for cells treated different concentrations. Negative control (media), Positive control (0.1μg/mL minoxidil) (b) Representative microscopic images of scratch assay for 24h. Data are presented as means ± standard error of the mean (SEM) (*$p < 0.05$, **$p < 0.01$)

Conclusion.

Our findings suggest that H-EVs represent a new candidate of hair loss treatment derived from *H. cordata* that may be used to promote hair growth and prevent hair loss. H-EVs exhibit a strong radical scavenging activity compared to the extracts of *H. cordata* in DPPH assays. The results confirm that H-EVs has high antioxidant activity and HFDP cell proliferative effect on HDP cells *in vitro*. Oxidative stress may be a pivotal mechanism contributing to the leading cause of hair loss, and dermal papilla cells were susceptible to oxidative stress and resulting in miniaturization of hair follicles. These results demonstrated that HFDP cell exposure to H-EVs accelerated cell migration. Taken together, these results suggest that H-EVs treatment induce cell migration and increase cell proliferation in HFDP cells. On the basis of our results, we propose that H-EVs could be implemented as an active ingredient for pharmaceutical or cosmeceutical applications. To ensure hair growth promoting activity of H-EVs via decreasing oxidative stress, further studies are required to understand the effects of bioactive compounds from H-EVs on specific molecular pathways.

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Conflict of Interest Statement.

NONE.

References.

[1] K. Kim, J. Park, J.-H. Jung, R. Lee, J.-H. Park, J.M. Yuk, H. Hwang, J.H. Yeon, Cyclic tangential flow filtration system for isolation of extracellular vesicles, APL bioengineering 5(1) (2021) 016103.

[2] K. Kim, Y.J. Sohn, R. Lee, H.J. Yoo, J.Y. Kang, N. Choi, D. Na, J.H. Yeon, Cancer-associated fibroblasts differentiated by exosomes isolated from cancer cells promote cancer cell invasion, International journal of molecular sciences 21(21) (2020) 8153.

[3] K. Kim, J. Park, Y. Sohn, C.-E. Oh, J.-H. Park, J.-M. Yuk, J.-H. Yeon, Stability of Plant Leaf-Derived Extracellular Vesicles According to Preservative and Storage Temperature, Pharmaceutics 14(2) (2022) 457.

[4] A.T. Reiner, V. Somoza, Extracellular vesicles as vehicles for the delivery of food bioactives, Journal of agricultural and food chemistry 67(8) (2019) 2113-2119.

[5] P. Pérez-Bermúdez, J. Blesa, J.M. Soriano, A. Marcilla, Extracellular vesicles in food: Experimental evidence of their secretion in grape fruits, European Journal of Pharmaceutical Sciences 98 (2017) 40-50.

[6] S. Raimondo, F. Naselli, S. Fontana, F. Monteleone, A.L. Dico, L. Saieva, G. Zito, A. Flugy, M. Manno, M.A. Di Bella, Citrus limon-derived nanovesicles inhibit cancer cell proliferation and suppress CML xenograft growth by inducing TRAIL-mediated cell death, Oncotarget 6(23) (2015) 19514.

[7] J. Mu, X. Zhuang, Q. Wang, H. Jiang, Z.B. Deng, B. Wang, L. Zhang, S. Kakar, Y. Jun, D. Miller, Interspecies communication between plant and mouse gut host cells through edible plant derived exosome-like nanoparticles, Molecular nutrition & food research 58(7) (2014) 1561-1573.

[8] R. Lee, H.J. Ko, K. Kim, Y. Sohn, S.Y. Min, J.A. Kim, D. Na, J.H. Yeon, Anti-melanogenic effects of extracellular vesicles derived from plant leaves and stems in mouse melanoma cells and human healthy skin, Journal of extracellular vesicles 9(1) (2020) 1703480.

[9] K. Kim, H.J. Yoo, J.-H. Jung, R. Lee, J.-K. Hyun, J.-H. Park, D. Na, J.H. Yeon, Cytotoxic effects of plant sap-derived extracellular vesicles on various tumor cell types, Journal of functional biomaterials 11(2) (2020) 22.

[10] K. Kim, J.-H. Jung, H.J. Yoo, J.-K. Hyun, J.-H. Park, D. Na, J.H. Yeon, Anti-Metastatic Effects of Plant Sap-Derived Extracellular Vesicles in a 3D Microfluidic Cancer Metastasis Model, Journal of functional biomaterials 11(3) (2020) 49.
[11] K. Shingnaisui, T. Dey, P. Manna, J. Kalita, Therapeutic potentials of Houttuynia cordata Thunb. against inflammation and oxidative stress: A review, Journal of ethnopharmacology 220 (2018) 35-43.
[12] S.J. Lee, W. Hu, E.J. Lee, J.Y. Choi, O.K. Koo, Polyphenolic Profile of Fermented Houttuynia cordata Thunb. and Overall Contribution to Antioxidant and Lipolytic Activities, Food Eng Prog 22(4) (2018) 295-303.
[13] J. Kim, J. young Shin, Y.-H. Choi, M. Jang, Y.J. Nam, S.Y. Lee, J. Jeon, M.H. Jin, S. Lee, Hair growth promoting effect of Hottuynia cordata extract in cultured human hair follicle dermal papilla cells, Biological and Pharmaceutical Bulletin 42(10) (2019) 1665-1673.
[14] R.M. Trüeb, Oxidative stress in ageing of hair, International journal of trichology 1(1) (2009) 6.
[15] H.J. Park, G.-R. Jin, J.H. Jung, S.B. Hwang, S.H. Lee, B.-H. Lee, Hair Growth Promotion Effect of Nelumbinis Semen Extract with High Antioxidant Activity, Evidence-Based Complementary and Alternative Medicine 2021 (2021).
[16] V. Vivcharenko, M. Wojcik, A. Przekora, Cellular response to vitamin C-enriched chitosan/agarose film with potential application as artificial skin substitute for chronic wound treatment, Cells 9(5) (2020) 1185.
[17] O. Kwon, J. Han, H. Yoo, J.-H. Chung, K. Cho, H. Eun, K.H. Kim, Human hair growth enhancement in vitro by green tea epigallocatechin-3-gallate (EGCG), Phytomedicine 14(7-8) (2007) 551-555.
[18] R.H. Kwon, B.J. Ha, Increased flavonoid compounds from fermented Houttuynia cordata using isolated six of Bacillus from traditionally fermented Houttuynia cordata, Toxicological Research 28(2) (2012) 117-122.