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Hair Growth Promoting Effect of Urticadioica L

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Objectives: We investigated the effect of *Urticadioica L* extracts on hair growth by using in-vitro and ex vivo study methods. **Methods:** Human single hair follicle and dermal papilla cells obtained from scalp skin samples of healthy volunteers. We evaluated the effect of *Urticadioica L* on hDPCs and on ex vivo hair follicle organ culture. Hair follicle matrix cell's proliferation marker Ki-67 identified by immunoflurescence staining. **Results:** *Urticadioica L* ethanol extracts promoted elongation of the hair shaft and reduced catagen transition of human hair follicles in organ culture model. E.extract of *Urticadioica L* increased Ki-67 positive matrix keratinocytes. **Conclusions:** *Urticadioica L* ethanol extract enhanced human hair growth in ex vivo organ culture model. Future study is needed related to its mechanism of hair growth.

Keywords: Dermal Papilla Cells (hDPCs), Hair Follicle, Urdicadioica L, Ki-67

Introduction

Stinging nettle (*Urticadioica L*) belongs to the family *Urticaceae* [1]. About 50 species of Stinging nettle (*Urticadioica*) are grown in the world. Three species of *Urticaceae* (*Urtica cannabina, Urtica angustifolia, Urtica dioica L*) grow in Mongolia [2]. *Urticadioica L* is a herbaceous perennial flowering plant that native to Europe, Asia, northern Africa and North America. The maximum typical height of this plant species ranges from 2 to 4 meters [3]. It produces pointed leaves and white to yellowish flowers [4]. The plant has many hollow stinging hairs called trichomes on its leaves and stems, which act like hypodermic needles, injecting histamine and other chemicals that produce a stinging sensation when contacted by humans and other animals [3]. Flavonoids, tanins, scopoletin, sterols, fatty acids, polysaccharides, isolectins andsterols are phytochemicals which are reported from this plant [5].

The whole plant is used in folk medicine to treat allergies, kidney stones, burns, anemia, rashes, internal bleeding, diabetes, etc. However only a few of these pharmacological activities have been experimentally proved [6]. Stinging nettle extracts has been different studies reported to have various pharmacological antioxidant, anti-microbial, anti-inflammatory, anti-ulcer, anticolitis and analgesic [7, 8]. As well as this plant

has been also used in human nutrition such as food or tea and also gathered commercially due to high content of chlorophyll, which is used as green coloring agent (E140) in food and medicines [5]. Despite the fact that science has not been proven its effectiveness, Mongolians have been using extracts from this plant to prevent hair loss and as a hair loss treatment.

Hair loss problem have approximately 2% of the world population [9]. According French scientists, one out of every five people have hair loss [10]. Depending on the clinical form, approximately 78% of men and 57% of women over the age of 80 are affected by androgenetic alopecia and 58% of men over the age of 50 are affected [11]. In Mongolia, the prevalence of hair loss has not yet been studied, but between in 2011-2015 a total of 1325 hair loss cases diagnosed in out-patient clinic of National Dermatology Center of Mongolia.

The hair follicle (HF) is a very small-organ [12]. In normal hair growth, the hair follicles cycle through successive phases: anagen phase of growth, catagen phase of involution, and telogen phase of rest [13]. The hair grown from the follicle will pass through the 4 stages of hair cycle to repeat hair growth and loss [12]. When generalized loss is present it is useful to assess the ratio of anagen (growing) hair to that of the telogen hair. In hair loss, there appears to be a dysfunction and disproportion in hair cycling, resulting in a reduced length of anagen phase, increased proportion of hair in catagen/telogen phase [14]. Many various factors including stress as social, psychological and mental, eating habits, and living habits, drugs etc can affect hair loss [15]. The most common form of human hair loss is androgenic alopecia.

Alopecia has few physically harmful effects, but may lead to psychological consequences, including high levels of anxiety and depression [16]. However, only two drugs so far have been approved for hair loss treatment by the Food and Drug Administration (FDA), finasteride and minoxidil [11]. Researchers are still studying low-cost therapeutic methods that are effectives against hair loss.

This study aimed to determine the effect of *Urticadioica L* on human hair growth. To accomplish this, we investigated the effect of *Urticadioica L* extracts on hair growth by using in-vitro and ex- vivo study methods.

Material and Method

Ethical statements

Study protocols were approved by the institutional research board of Mongolian National University of Medical Sciences [IRB approval number], and written informed consent was obtained from all subjects. All experimental procedures using human tissues were performed according to the principles described in the declaration of Helsinki.

Human hair follicle samples

A total of 479 human scalp HFs were obtained from 7 different healthy male volunteers 20 to 40 years of age without current or prior scalp diseases. The samples were obtained from 1.5x1.0 cm of scalp from their occipital region yielding greater than 60 HFs per subject. The samples were carefully dissected into single HFs under a stereo microscope (Motic K Series). In this study, only anagen HFs were used.

Preparation of Plant extract

The aerial part of the *Urticadioica L* was harvested according to the standards of "A" Pharmaceutical Factory (Ulaanbaatar, Mongolia). *Urticadioica L* extract was obtained by adding water, ethanol, hexane as solvents to macerated method *Urticadioica L* at the room temperature for 72 hours [17]. The solvent and extraction ratio were same (1:10). After filtration through filter paper, the filtrates were concentrated and dried by vacuum evaporation and freeze drying method.

Cell culture

As previously described [18, 19], the hair dermal papilla cells (hDPCs) were cultured at 37°C with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM; Welgene, Daegu, Korea), supplemented with 10% fetal bovine serum (Welgene), 10 ng/ml basic fibroblast growth factor (bFGF; R&D Systems, Minneapolis, MN, USA), and antibiotic/antimycotic 1x solution (100 mg/ml streptomycin and 100 U/ml penicillin).

Thiazolylblue (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazoliumbromide) (MTT) assay

Cell viability was measured by the MTT assay. Cells at $1x10^4$ cellsper well were seeded into 96-well plates, serum-starved for 24 hours, and then treated for 24 hours with the vehicle

(ethanol at a diluted to 1:1,000 in serum-free DMEM) or with *Uricadioica L* extracts (0.125 mg/ml, 0.25 mg/ml, 0.5 mg/ml [final concentration], diluted to 1:1,000 in serum-free DMEM). Subsequently, 15 µl of MTT solution was added to each well, and the plate was incubated for 4 hours at 37°C. The supernatant containing the MTT was removed, and the formazan crystals in the wells were dissolved by adding 100 µl of dimethyl sulfoxide. The samples were finally incubated for 30 minutes at room temperature before a plate reader enabled quantitative analysis for viable cells by optical density measurement at 570 nm.

Human hair follicles organ culture and immunofluorescence staining

Isolated human scalp hair follicles were cultured as described previously [20, 21]. Each HF was cut at the level of the sebaceous duct, and then cultured for 12 days at 37°C (5% CO_2) in Williams' E medium (Gibco-BRL laboratories, Grand Island, NY, USA), supplemented with 10 ng/ml hydrocortisone, 10 µg/ml insulin, 2 mM L-glutamine, and antibiotic/antimycotic 1x solution (penicillin and streptomycin, Gibco-BRL laboratories). *Urticadioica L* water, hexane and ethanol extracts was added to culture medium at final concentrations of 0.125 mg/ml. At every third day, elongation of the hair shaft was measured, and HFs in anagen phase were determined according to their growth.

Immunofluorescence staining was performed to evaluate proliferation of HF matrix keratinocytes. As previously described, immunoreactivity for Ki-67 (DAKO, Carpinteria, CA, USA) was used as an indicator of cell proliferation [22]. A DAPI mounting media kit (Vector Laboratories, Burlingame, CA, USA) was used to counterstain the nuclei.

Statistical analysis

Statistical significance was determined using Student's t-test. Paired t-test was used to compare the HF culture results. All tests

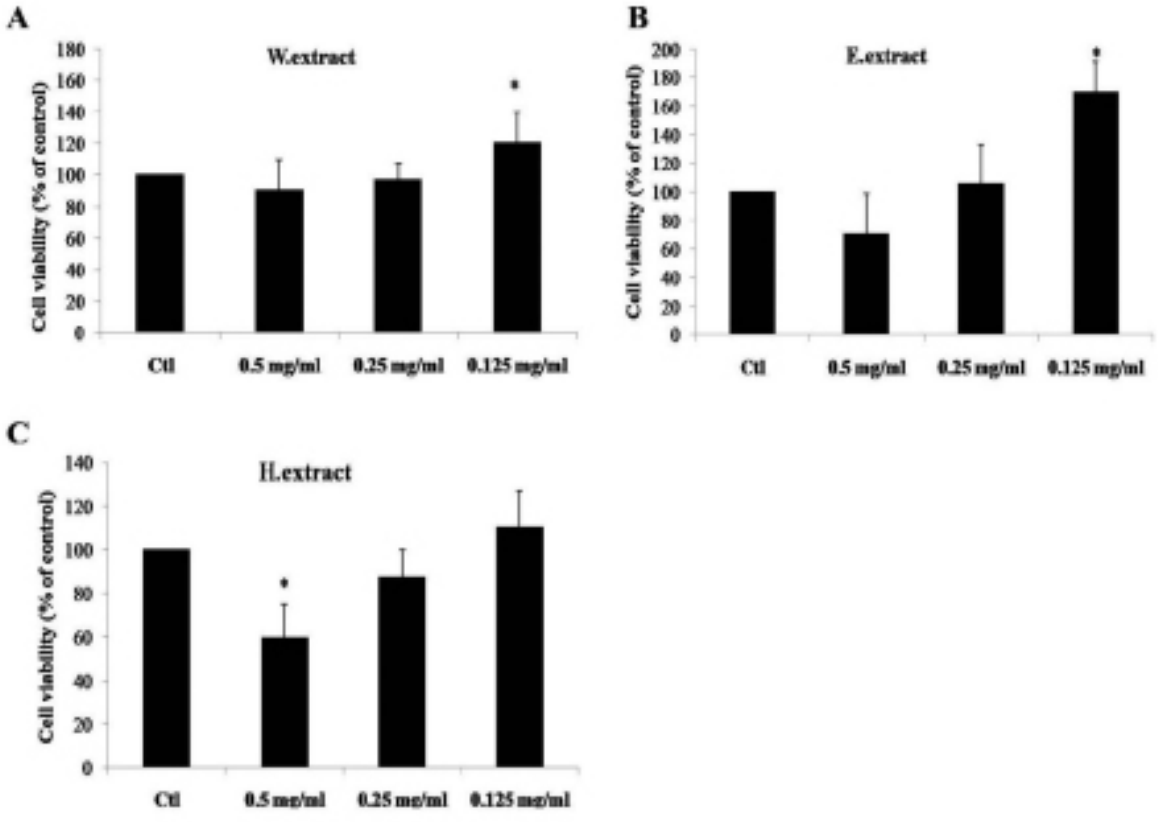

Figure 1. The viability of cultured human dermal papilla cells (hDPC's) on treatment of *Urdicadioica L* extracts. Cells were treated with *Urticadioica L* extracts, or vehicle for 24 hours. (A) water extract (W.extract) of *Urticadioica L*, (B) ethanol extract (E.extract), and (C) hexane extract (H.extract). The results are expressed as mean ± standard error.

were two-tailed, and differences with a p-value of <0.05 were considered statistically significant. The statistical analyses were done by using the STATA 13 software package.

Results

Effect of Urticadioica L extracts on DPC proliferation

We performed the MTT assay to evaluate the effect *Urticadioica L* extracts on hDPCs' survival. In this experiment, *Urticadioica L* water, ethanol, hexane extracts (0.125 mg/ml) significantly enhanced viability of hDPCs compared to the vehicle-treated controls. However, the hDPCs' viability decreased at all extracts concentrations that were greater than 0.250 mg/ml (Figure 1 A,B,C).

Effect of *Urticadioica L* extracts on matrix cell proliferation and hair shaft elongation in cultured human hair follicles

We performed in vitro culture of the healthy human scalp HFs to examine the effect of *Urticadioica L* extracts at the organ level. Hair shaft elongation by treating culturing human HFs with 0.125 mg/ml concentration of *Urticadioica L* water, ethanol and hexane extracts for 12 days was analyzed. HFs treated with *Urticadioica L* ethanol extract significantly enhanced hair shaft elongation after 6, 9, and 12 days, compared to the vehicle-treated controls. As well, *Urticadioica L* hexane extract grew longer than HFs treated with vehicle (Figure 2A). *Urticadioica L* ethanol extract reduced catagen transition compared to the vehicle control and other extracts (Figure 2B).

To analyze the proliferation of human HFs matrix cells, immunofluorescence staining was performed for Ki-67 (a proliferation marker) after HFs were cultured with 0.125 mg/ml concentration of *Urticadioica L* water, ethanol and hexane extracts for 3 days. The number of Ki-67+ matrix keratinocytes were counted and normalized by using the number of DAPI positive cells. We found that treatment with *Urticadioica L* ethanol extract (0.125 mg/ml) significantly increased Ki-67+ matrix keratinocytes than vehicle control (Figure 3A and B).

Discussion

The hair follicle (HF) is composed of epidermal (epithelial) and dermal (mesenchymal) compartments and their interaction has an important role in the morphogenesis of hair, growth of the hair follicle, and the hair cycle [23]. The dermal papilla, composed of specialized fibroblasts located in the bulb of the hair follicle, contributes to the control of hair growth and the hair cycle [21].

Single follicle organ culture method, which is an ex vivo model for the study of freshly isolated human scalp HFs under serum-free conditions developed by Philpott et al, [22]. This

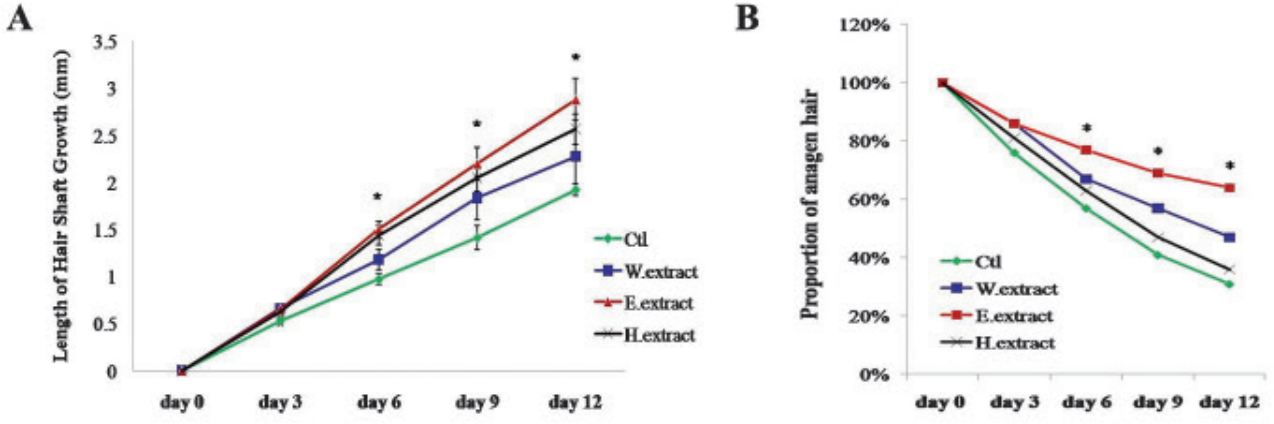

Figure 2. The effect of *Urticadioica L* extracts onhair shaft elongation and catagen transition in ex vivo hair follicle organ culture. HF's cultured with 0.125 mg/ml concentration of *Urticadioica L* water, ethanol and hexane extracts for 12 days (n = 7). (A) The effect of E.extract of *Urticadioica L* on hair shaft elongation and (B) The effect of E.extract of *Urticadioica L* oncatagen transition as compared to the vehicle control. The results were expressed as mean ± standard error. * $p \leq 0.05$, vs. the control group. Ctl: Control, W.extract: Water extract of *Urticadioica L*, E.extract: Ethanol extract of *Urticadioica L*, H.extract: Hexane extract of *Urticadioica L*.

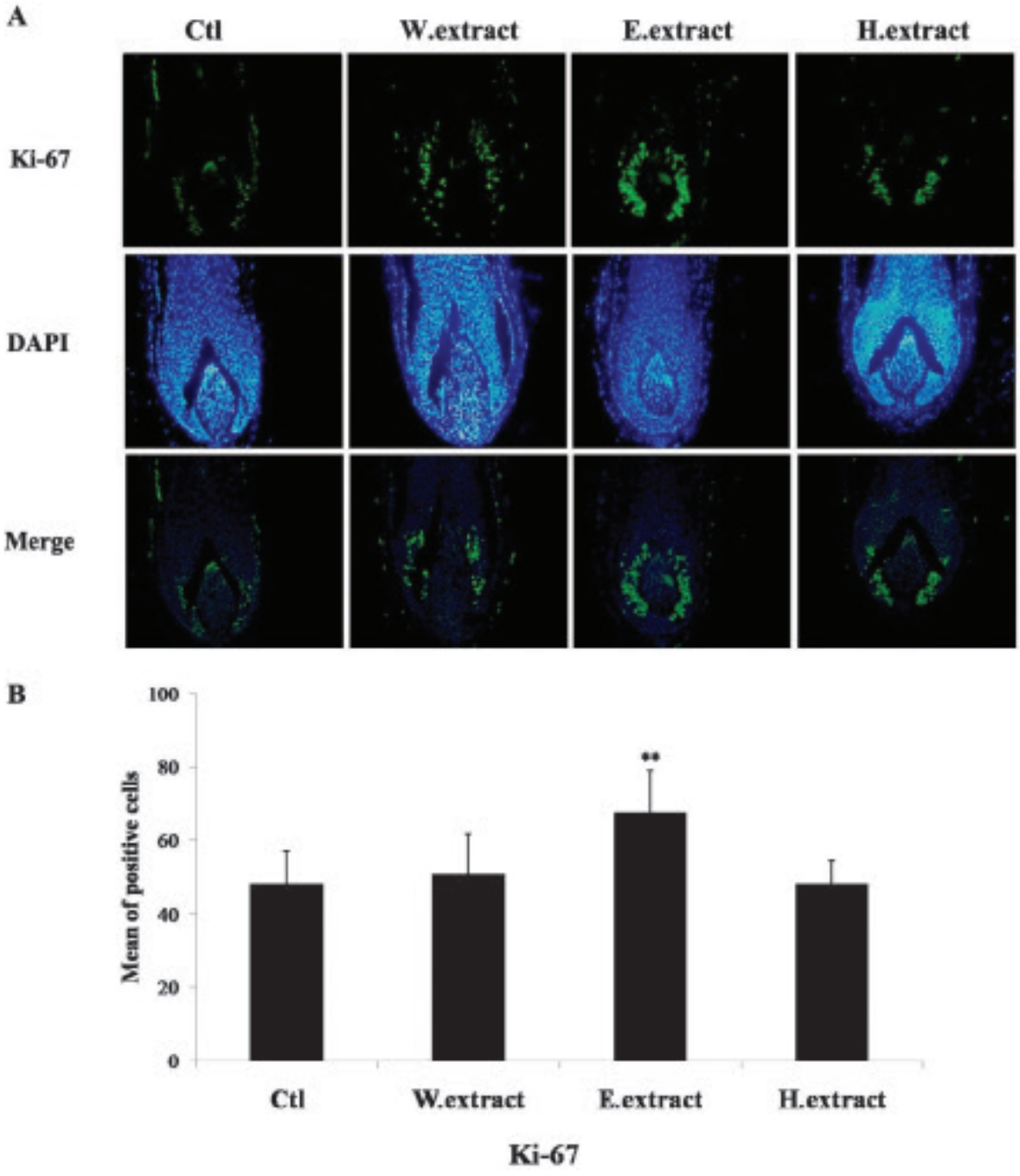

Figure 3. The proliferation of matrix keratinocytes in *Urticadioica L* ethanol extract treated HFs. (A) Human HFs were cultured with the vehicle or water, ethanol and hexane extracts (0.125 mg/ml) for 3 days, and then subjected to immunofluorescence staining, to examine proliferation in the hair matrix keratinocytes, with Ki-67 (proliferation, green fluorescence), and 4',6-diamidino-2-phenylindole (DAPI, blue fluorescence) to counterstain the nuclei (X200). (B) For quantitative analysis, Ki67+ cells were counted and normalized to DAPI-stained cells. E.extract of *Urticadioica L* increased proliferation in the hair matrix keratinocytes. The results were expressed as mean ± standard error. * $p \leq 0.05$, **$p \leq 0.01$, vs. the control group. Ctl: Control, W.extract: Water extract of *Urticadioica L*, E.extract: Ethanol extract of *Urticadioica L*, H.extract: Hexane extract of *Urticadioica L*.

method, not only compares the single hair shaft elongation and hair cycle change within the study groups and also has the advantage using immunohistochemical and immunofluorescence staining evaluate the desired markers related proliferation and differentiation [24].

Ki-67 immunoreactivity (IR) is a useful tool to obtain information on cell proliferation in situ, with the significant advantage that it can be used on both cryo and paraffin embedded sections [25]. The changes in Ki-67 IR during the hair cycle transition are well demonstrated. During anagen VI, Ki-67 IR is most prominent in hair matrix keratinocytes and the onset of catagen is marked by the reduction in the percentage of Ki-67+ matrix keratinocytes, and no Ki-67+ keratinocytes present in late catagen [26-28]. Therefore, in our study we also hair shaft elongation and examined Ki-67 IR by immunofluorescence staining.

Urticadioica L elaborates different classes of organic compounds of medicinal importance including phytosterols, saponins, flavanoids, tannins, sterols, fatty acids, carotenoids, chlorophylls, proteins, amino acids, macro, micro-elements and vitamins [5].

In our study, we confirmed that *Urticadioica L* ethanol extracts enhanced hair shaft elongation, reduced catagen transition and significantly increased the Ki-67+ matrix keratinocytes compared with the vehicle control and water and hexane extracts.

Previous research has shown the *Urticadioica L* chemical components which are mainly soluble in ethanol are flavonoid, polyphenol, lectins, sterol, lignans, beta carotene, keton, palmitic and steric acid, oleic acid, etc [29]. Polyphenols and some flavonoids has been reported to hair growth promoting effect by the proliferation of hDPCs with ROS scavenging and increasing of growth factors IGF-1 and VEGF, also confirmed hair re-growth promotion in mice [30-32].

Our study has some limitation. First, we used only whole plants and single proliferation marker Ki67. Using additional proliferation marker could make our study more effective. Second, our sample size was small.

Further researches are required for finding the effective molecules or compounds and related mechanism.

We conclude above mentioned components such as polyphenols and flavonoids which are soluble in ethanol of *Urticadioica L* may promoteon hair growth.

Conflict of Interest

The authors state no conflict of interest.

Acknowledgement

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