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Review

Study on the Effect of *Cimicifuga heracleifolia* Ethanol Extract on Hyaluronic Acid Synthesis

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Abstract

Hyaluronic acid (HA) is a mucopolysaccharide, occurring naturally in living organisms. It is one of the most hydrophilic molecules, so it has been known as being related to skin hydration and skin aging. The purpose of this study was to examine the effects of *Cimicifuga heracleifolia* ethanol extract on the hyaluronic acid synthesis and the inhibition of hyaluronidase activity. To determine cytotoxicity, hyaluronic acid synthase 2 (HAS2) gene expression, HA production and, hyaluronidase inhibitory effects, 3-(4,5-dimethylthiazol-2-ly)-2,5-diphenyl tetrazolium bromide (MTT) assay, real time - polymerase chain reaction (RT-PCR), hyaluronic acid enzyme linked immunosorbent assay (HA-ELISA), and hyaluronidase assay were used, respectively. When the *Cimicifuga heracleifolia* extract was treated in the HaCaT cells up to 500 μg/mL concentration, cytotoxicity was confirmed by the *Cimicifuga heracleifolia* extract at concentrations above 200 μg/mL. Therefore, the optimum concentration of all experiments used in this study was determined to be 200 μg/mL. HAS2 gene expression increased by *Cimicifuga heracleifolia* extract in a concentration-dependent manner at all treatment concentrations. The production rate of HA was tended to decrease at the highest concentration of 200 μg/mL. The hyaluronidase activity inhibition effect of *Cimicifuga heracleifolia* extract was very high compared to the control group. Based on these results, *Cimicifuga heracleifolia* extract was expected to have a moisturizing effect on human skin and special attention should be paid to the determination of the concentration of *Cimicifuga heracleifolia* when developing cosmetic materials using it.

***Keywords**: Cimicifuga heracleifolia, Hyaluronic acid (HA), Hyaluronic acid synthesis 2 (HAS2), Hyaluronidase, HaCaT cell*

1. Introduction

Among the various tissues that make up the human body, the skin is a dynamic tissue that has an extensive surface area and undergoes continued cycles of cell division, proliferation, and exfoliation during a human lifetime[1]. The skin comprises epidermal, dermal, and subcutaneous layers. Sweat glands, pores, and sebaceous glands, which are distributed throughout the epidermal layer, play key roles in regulating the hydration-related metabolism of cells[2]. To maintain healthy skin, the stratum corneum must contain at least 10% moisture, for which various types of lipids, sugars, and amino acids are known to be involved[3,4]. Among these, hyaluronic acid (HA), a type of glycosaminoglycan present in the extracellular connective tissue, is known to function as a barrier that prevents the evaporation of moisture from the skin[5]. Physiologically, levels of HA are known to decrease with increasing age. Furthermore, exposure to excessive UV levels is typically associated with decreases in HA, along with factors such as a dry environment, excessive stress, smoking, and consumption of alcohol[6,7]. Decreases in the HA content of skin tissue can, in turn, result in reduced skin elasticity, dry skin, and wrinkles. Therefore, maintaining the HA content of skin tissue at a certain level or above is an important element of skin moisturizing and beauty treatments[8]. Substances that induce the synthesis or inhibit the decomposition of HA are effective in moisturizing skin and preventing skin aging, and in this regard, among the various hyaluronic acid synthase (HAS), HAS2 and HAS3 are known to play important roles in the synthesis of HA. Accordingly, many studies have focused on enhancing the production of HA through the increased expression of HAS genes[2,9].

Cimicifuga heracleifolia is a wind-heat expelling drug derived from the dried roots and rhizomes of perennial plants belonging to the Ranunculaceae family, including *Cimicifuga heracleifolia* Komarov, *Cimicifuga dahurica* (Turcz.) Maximowicz, and *Cimicifuga foetida* Linne.[10]. *Cimicifuga heracleifolia* was mentioned for the first time in SinNongBonChoGyeong (神農本草經) is a high-quality product, and owing to its detoxification efficacy, it has been used for various dermatological and febrile diseases. *Cimicifuga heracleifolia* also referred to as ju-ma (周麻), Gwan-Seung-Ma (關升麻), Yong-An-Geun (龍眼根), Go-Ryeok-A (苦力芽). It is found throughout various regions in Asia, including the Korean Peninsula[11]. Recent studies have provided scientific evidence of the antibacterial and anti-inflammatory effects of *Cimicifuga heracleifolia*, and major pharmacologically active components of which are known to be actein, cimisides A-F, cimicifugic acid,

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cimicifugin, and esculetin[12,21]. The three species of *Cimicifuga* from which the herbal medicine *Cimicifuga heracleifolia* is derived have the same efficacy and temperament. Therefore, they are often used as mixtures in clinical practice without differentiating one from the other. Their temperament is mildly cold and the taste is somewhat bitter, their oriental medicine effects generally target the lungs, spleen, and gastrointestinal system[12]. In clinical practice, the efficacy of *Cimicifuga heracleifolia* in clearing rashes and detoxification is used in the treatment of wind-fever headache, sore throat, and Yang poison exanthema[13]. Moreover, under their efficacy in elevating Yang qi (陽氣), these drugs are known to be qi (氣)-lifting agents used for symptoms that involve the physical deterioration of organs due to the lack of qi (氣), such as gastroptosis, metroptosis, and proctoptosis[12,13]. In terms of dermatological diseases, *Cimicifuga heracleifolia* has therapeutic effects on rashes caused by wind-heat or heat poison, which is expected to be linked to modern moisturizing effects. In Traditional Korean Medicine, *Cimicifuga heracleifolia* has been frequently used to obtain skin moisturizing effect through vital blood, SaengHyeolYunBuEum (生血潤膚飮), SeungMaByeolGapTang (升麻鼈甲湯), YunHyeolYum (潤血飮) and DangGuiHwaHyeolTang (當歸和血湯) was used. Accordingly, we conducted an *in vitro* efficacy assessment of the skin moisturizing effect of *Cimicifuga heracleifolia* and obtained significant results, so we want to report this study.

2. Experimental

2.1. Materials

2.1.1. Cell culture and experimental conditions

The HaCaT cell line used in this study was an immortalized human keratinocyte cell procured from ATCC (USA). HaCaT cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) containing 10% fetal bovine serum (FBS, Gibco) and 1% penicillin-streptomycin (PS, Gibco) at 37 °C in a 5% CO_2 atmosphere.

2.1.2. Reagent

Cimicifuga heracleifolia was purchased from Pure Mind Pharmaceuticals (YeongCheon, Gyeongsangbuk-do, Korea), the samples of which were stored in Seowon university central laboratory. Medicinal herb (100 g) was added to 70% ethanol (v/v) solvent for extraction and collected in a bottle after filtering. This process was repeated three times. After concentrating on a rotary vacuum evaporator, the samples were used as freeze-dried preparations in the experiment.

2.2. Methods

2.2.1. Hyaluronidase inhibition assay

To measure the hyaluronidase activity inhibition rate, we performed a hyaluronidase enzyme assay in accordance with the Morgan-Elson method. After mixing 25 μL of bovine hyaluronidase (7.9 mg/mL in 0.1 M acetate buffer, pH 3.6) with 25 μL of the sample, the mixture was incubation for 20 min at 37°C. After adding 100 μL of $CaCl_2$ (12.5 mM), the mixture was incubation for a further 20 min at 37°C. Next, 125 μL of sodium hyaluronate (12 mg/5 mL in 0.1 M acetate buffer, pH 3.6) was added and the mixture was incubation for 40 min at 37°C, after which 50 μL of NaOH (0.4 M) and 50 μL of potassium tetraborate (0.4 M) was added. The mixture was placed in boiling water for 3 min and then cooled at room temperature. after which, the DMAB solution (750 μL) was added and incubation for 20 min at 37°C. Subsequently, absorbance at 585nm was measured and the results were compared with those obtained for, a known hyaluronidase inhibitor. The inhibition rate was calculated using the following formula.

$$\text{Inhibition rate (\%)} = \frac{OD_{Control} - OD_{Sample}}{OD_{Control}} \times 100 \quad (1)$$

$OD_{Control}$: Absorbance with hyaluronidase only

OD_{Sample} : Absorbance of *Cimicifuga heracleifolia* extract treatment group

2.2.2. Cytotoxicity assay

Cell viability assay was performed using HaCaT cells to observe skin cell toxicity by *Cimicifuga heracleifolia*. HaCaT Cells were seeded in 96-well plates at a concentration of 1.5×10^4/well and culturing for 24 h. After changing the medium to serum-free DMEM, the cells were treated with diluted *Cimicifuga heracleifolia* extract (50, 100, 200 and 500 μg/mL). After culturing for 24 h, the medium was discarded, after which 20 μL of 3-(4,5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT, 5 mg/mL) was added and the cells were cultured for 2 h in a CO_2 incubator. After dissolving the formazan crystals with 100 μL of DMSO, the absorbance at 570 nm was measured. Cell viability was expressed as a percentage relative to control cell viability. The final concentration of DMSO in the treated cell samples was set not to exceed 0.1%.

2.2.3. RT-PCR

Cells were seeded in 6-well plates at a concentration of 3.0×10^5/well and culturing for 24 h. After changing the medium to serum-free DMEM, the cells were treated with *Cimicifuga heracleifolia* extract (50, 100 and 200 μg/mL) with a final DMSO concentration of 0.1%. After culturing for 24h, total RNA was extracted using an easy-Blue RNA extraction kit (Intron). To 1 ml of easy blue of chloroform was added and precipitated for 5 min. The cell lysates were centrifuged for 10 min at 4°C 13,000 rpm. The supernatant liquid was collected 450 μl and transferred to an empty 1.5 ml conical tube. The equal volume of isopropanol was added to the 1.5 ml conical tube containing supernatant liquid and incubated for 10 min. The tubes were centrifuged for 5 min at 4°C 13,000 rpm. The pellet was washed with 1 ml of 75% ethanol. After 75% ethanol removal, total RNA was dissolved in DEPC water. After measuring the concentration and purity of RNA (OD260/OD280) and measured 2μg of RNA, cDNA was synthesized using the power cDNA synthesis kit (Intron). A PCR premix kit (Solgent) was used for PCR. HAS2 gene production was identified from the bands appearing on 1.5% agarose gels. For the positive control, 1 μM of all-trans-retinoic acid (ATRA, Sigma) was used. The Image J program was used to measure the intensity of the band. Image

Table 1. Primers Sequence of HAS2 and GAPDH

Gene	Direction	Sequence (5' → 3')	Size (bp)
HAS2	Forward	GCT ACC AGT TTA TCC AAA CG (20 mer)	393
	Reverse	GTG ACT CAT CTG TCT CAC CG (20 mer)	
GAPDH	Forward	ATT GTT GCC ATC AAT GAC CC (20 mer)	546
	Reverse	AGT AGA GGC AGG GAT GAT GT (20 mer)	

The PCR conditions were as follows.
1 cycle - 94 °C 15 min
50 °C 30 sec, 72 °C 60 sec
32~35 cycles - 94 °C 30 sec
1 cycle - 72 °C 10 min

J is a JAVA-based image processing and analysis program, developed at the National Institutes of Health (NIH) and the Laboratory for Optical and Computational Instrumentation (LOCI, University of Wisconsin). This program is used in a wide variety of research fields. The primers used for amplification have been described below (Table 1).

2.2.4. HA-ELISA

Cells were seeded in 24-well plates at a concentration of 1.5 × 10^5/well and culturing for 24 h, the cells were washed twice with PBS. The medium was changed to serum-free DMEM and the cells were treated with *Cimicifuga heracleifolia* extract (50, 100 and 200 μg/mL). The final concentration of DMSO in the extract was set to 0.1%. After 24 h, discard the supernatant and centrifuged for 10 min at 12,000rpm. The samples were stored at 4 °C until ELISA was performed using an HA-ELISA kit (Echelon) following the manufacturer's protocol. As a positive control, 1 μM of ATRA was used.

2.2.5. Statistical analysis

For statistical analysis, Student's *t*-test was used with the significance level set to a p-value < 0.05.

3. Results

3.1. Effect of inhibition of hyaluronidase activity by *Cimicifuga heracleifolia* extract

The hyaluronidase inhibitory effect of *Cimicifuga heracleifolia* extract was much higher than that of many other natural products such as *Adenophora triphylla, Eucommia ulmoides*, and *Pueraria lobata*[22]. Although the hyaluronidase used in this study was not human-derived, it may still be used to indirectly determine hyaluronidase inhibition in the skin. When treated with a concentration of 200 μg/mL, *Glycyrrhiza uralensis* recorded an inhibition rate of 36.01%, whereas that attributable to *Cimicifuga heracleifolia* extract concentration of 200 μg/mL was 53.08% (Figure 1). Thus, *Cimicifuga heracleifolia* extract showed greater inhibition effect of hyaluronidase activity when compared with the *Glycyrrhiza uralensis*.

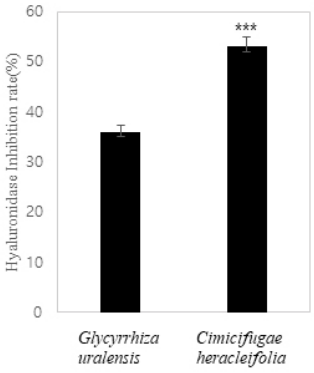

Figure 1. The inhibitory effect of *Cimicifuga heracleifolia* ethanol extract (200 μg/ml) on hyaluronidase activity. *Glycyrrhiza uralensis* ethanol extract (200 μg/ml) was used as a positive control. Values are represented as the percentage of hyaluronidase inhibition rate. Results are presented as mean ± S.D. of triplicate determinations. *** p < 0.001 each compared to control.

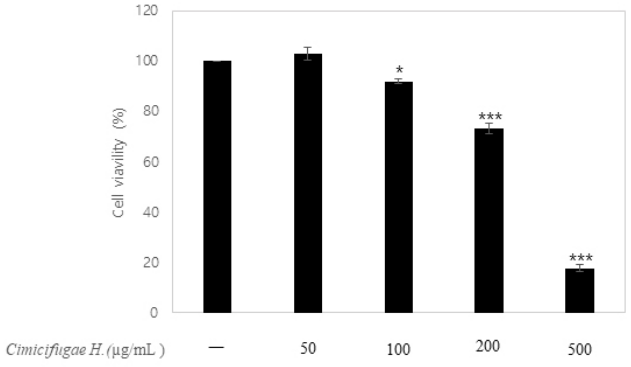

Figure 2. The effect of ethanol extract of *Cimicifuga heracleifolia* (*Cimicifugae H.*; 50, 100, 200 and 500 μg/mL) on HaCaT cell viability. Cell viability values are represented as the percentage relative to control. Results are presented as mean ± S.D. of triplicate determinations. * p < 0.05 and *** p < 0.001 each compared to control.

3.2. Cytotoxicity of HaCaT cells by *Cimicifuga heracleifolia* extract

The MTT assay was performed to determine the cytotoxicity of 70% ethanol extract of *Cimicifuga heracleifolia*. HaCaT Cells treated with *Cimicifuga heracleifolia* ethanol extract at concentrations of 50, 100, 200, and 500 μg/mL were expressed viability 102.72%, 91.99%, 73.06%, and 17.72% respectively. Although a slight decrease in cell viability was found at concentrations over 200 μg/mL, there was no pronounced cytotoxic effect (Figure 2).

3.3. Increased expression of the HAS2 gene by *Cimicifuga heracleifolia* extract

RT-PCR was performed to examine the expression of the HAS2 gene in cells treated with a 70% ethanol extract of *Cimicifuga heracleifolia*. The groups treated with the *Cimicifuga heracleifolia* extract showed expression of the HAS2 gene at all concentrations, among which the group exposed to a concentration of 200 μg/mL showed the strongest band (Figure 3A). The band intensity was measured using the

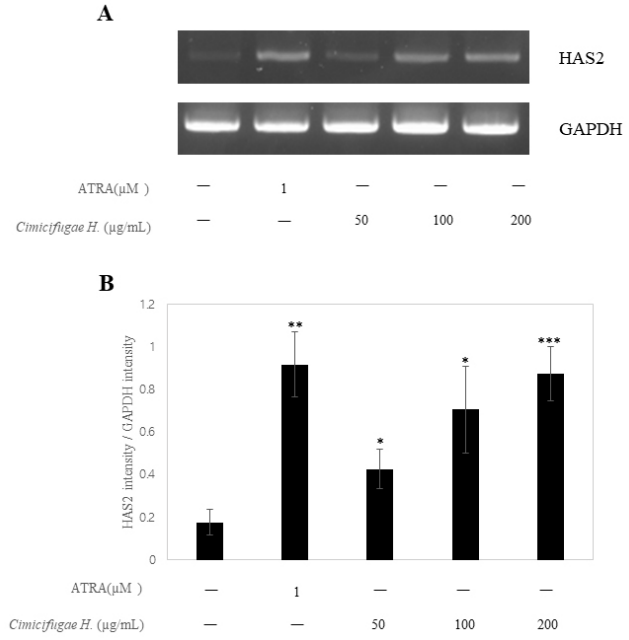

Figure 3. The effect of ethanol extract of *Cimicifuga heracleifolia* (*Cimicifugae H.*; 50, 100, and 200 μg/mL) on HAS2 gene expression in HaCaT cells. RT-PCR band of HAS2 and GAPDH (A) and the ratio of HAS2 to GAPDH band intensity (B). Results are presented as mean ± S.D. of three different experiments. All-trans-retinoic acid (ATRA) was used as a positive control. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ each compared to control.

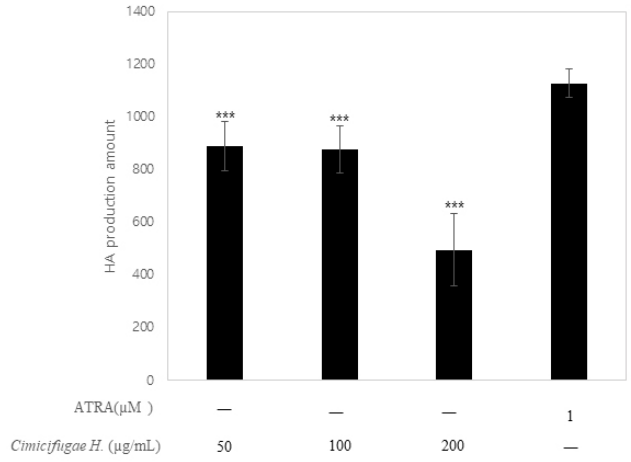

Figure 4. The effect of ethanol extract of *Cimicifuga heracleifolia* (*Cimicifugae H*; 50, 100 and 200 μg/mL) on HA production of HaCaT cells. HA concentration was measured using the HA-ELISA kit. Results are presented as mean ± S.D. of three different experiments. All-trans-retinoic acid was used as a positive control. ***$p < 0.001$ each compared to the positive control.

Image J program and it is shown in the graph (Figure 3B). Based on these results, it was determined that an ethanol extract of *Cimicifuga heracleifolia* is effective in inducing the expression of a gene involved in HA synthesis in human skin-derived keratinocytes.

3.4. Increased expression of the HA by *Cimicifuga heracleifolia* extract

HA-ELISA was performed to determine the level of HA produced in cells when treated with 50, 100, or 200 μg/mL of a 70% ethanol extract of *Cimicifuga heracleifolia*. When the level of HA production in the experimental groups was measured at 30 min, cells treated with extract concentrations of 50, 100, and 200 μg/mL were found to have produced 887.98, 875.44 and 494.78 ng/mL HA, respectively (Figure 4). As the results show, HA production decreased as the extraction concentration increased. Increased of HAS2 gene expression was confirmed in the RT-RCR, but could not observe a concentration-dependent increase in HA accorded to the HA-ELISA. These results are judged that the *Cimicifuga heracleifolia* extract had a specific effect on the HA biosynthesis pathway or was cytotoxicity reaction of natural extracts on HaCaT cells. In this regard, future studies should aim to identify the route of HA expression.

4. Discussion and Conclusions

The human skin not only functions to protect the internal organs from external stimuli and pollutants but is also known to play a very important immunological role[14]. Various skin functions can be lost or weakened by exposure to external stimuli or due to natural aging, which can cause a further acceleration of skin aging or have an effect on normal bodily metabolism[6,7,15]. External factors, such as dry air, UV light, visible light, and radiative heat, interfere with normal metabolism in the epidermal and dermal layers and also inhibit cell division, which can promote a reduction in skin regeneration, onset of skin cancer, inhibition of vitamin D synthesis, impairment of immune functions, and reductions in antioxidative and defense functions[16]. Decreased skin elasticity and an increase in wrinkles are most closely associated with decreases in the levels of collagen that is found mostly throughout the dermal layer and is also known to have a certain association with the denaturation and loss of glycosaminoglycans (GAGs), which are substances that bind various intracellular matrices[16,17].

HA, a GAG that is found mostly throughout the skin, is a long straight-chain macromolecule with a molecular weight of 200,000~400,000, which comprises β-d-glucuronic acid and β-d-*N*-acetylglucosamine monomers linked by beta bonds. It is widely distributed in the extracellular matrices of tissues and binds with moisture to form a gel, which enables it to hold moisture up to more than 1,000 times its weight. Consequently, it has been reported to be involved in retaining moisture within tissues, maintaining intercellular distance, cell division, differentiation, and migration, storage and dispersion of cell growth factors and nutrients, and immune regulation[2,16,18,19]. Among human cells, HA is mainly found in skin cells, and because a decrease in HA is associated with skin aging, which is manifested by the development of wrinkles, loss of elasticity, and reduction in moisture content, it is considered to be a very important substance with respect to skin beauty and dermatological treatments. Accordingly, various methods for maintaining HA concentrations in skin cells have

been proposed. However, because it is difficult to deliver HA into the skin layers, methods for inducing the production of HA within cells have been investigated[9,20].

In the hyaluronidase inhibition experiment, *Cimicifuga heracleifolia* extract treated at 200 μg/mL concentration appeared 53.08% inhibition rate, which was higher than 36.01% of *Glycyrrhiza uralensis*. It has been reported that the amount of hyaluronic acid in human skin decreases with aging and the decrease in the amount of hyaluronic acid in the skin is considered to be one of the direct causes of deterioration of skin elasticity and water content with aging[23,24]. Therefore, the inhibition of hyaluronidase, a hyaluronic acid degrading enzyme, is expected to be a means of minimizing the decrease of skin elasticity and decrease of moisture content.

To determine the cytotoxicity of *Cimicifuga heracleifolia*, an MTT assay was performed by treating cells with extracts at concentrations of 50, 100, 200 and 500 μg/mL. The results showed that cell viability relative to control was 102.72%, 91.99%, 73.06%, and 17.72% at a concentration of 50, 100, and 200 μg/mL, respectively. Although cell viability decreased slightly when treated at a concentration of 200 μg/mL, the cytotoxic effect was not significant.

To determine the expression of the HAS2 gene in response to treatment with *Cimicifuga heracleifolia* extract, we performed RT-PCR. We detected HAS2 gene expression in all groups of cells treated with each different concentrations of a 70% ethanol extract of *Cimicifuga heracleifolia*, with those cells treated with 200 μg/mL of extract showing the strongest expression. These findings indicate that, within the treatment concentration range used in the present study, *Cimicifuga heracleifolia* can promote an increase in the HAS2 gene synthesis of skin keratinocytes.

Since the increase of HAS2 gene synthesis was confirmed in the results of the RT-PCR, the HA-ELISA was conducted to confirm the increase of HA in HaCaT cells. But the results of HA-ELISA showed that HA decreased with increasing concentration of *Cimicifuga heracleifolia* extract. Which either had a specific effect on the HA synthesis pathway or increased HA synthesis within the cell, but also needs to be considered the possibility that HA would not be ejected outside from the cell. It has been reported that hyaluronic acid synthesis in skin cells is increased by various growth factors, tans-retinoic acid, N-methylserine, etc[25,26,27,28,29,30,31] and that estradiol and similar substances applied to skin increase the synthesis of HA[32,33,34]. The precise mechanism of the metabolic process of hyaluronic acid is not studied yet. Just the synthesis of HA is carried out by HAS on the inner surface of the cell membrane, which is known to penetrate the cell membrane and accumulate in the extracellular matrix during synthesis[35]. Therefore, additional experiments such as measuring the amount of HA in the cells by lysing the cells separately from the supernatant in the cells treated with *Cimicifuga heracleifolia* extract and the study on the detailed mechanism of HA synthesis are considered necessary.

On the basis of the findings of the present study, we have established that *Cimicifuga heracleifolia* extract can have a relatively significant skin moisturizing effect. In this study, we examined the moisturizing effect of *Cimicifuga heracleifolia*, using extract concentrations of 50, 100, and 200 μg/mL. The results of the experiment showed an increase in the amount of HAS2 gene produced with increasing treatment concentration. We believe that various methods for exploiting the skin beauty and moisturizing effects of *Cimicifuga heracleifolia* could be developed through future studies based on comparisons of the moisturizing effects of traditional prescriptions, including *Cimicifuga heracleifolia*, with the effects of new ingredients.

Declarations

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

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