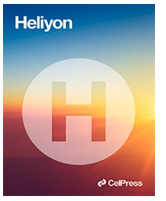
Heliyon 10 (2024) e24434

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Research article

# *Lespedeza maximowiczii* flower absolute promotes skin epithelization, barrier properties, and moisturization-related beneficial responses in human keratinocytes

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ARTICLE INFO

*Keywords:*
*Lespedeza maximowiczii*
Absolute
Skin re-epithelization
Skin barrier
Skin moisturizing
Keratinocyte

ABSTRACT

*Lespedeza maximowiczii* (LM), a member of the legume family, has tyrosinase inhibitory and estrogenic activities. However, its effects on skin-related biological activities remain unclear. Therefore, the present study aimed to explore the effects of LM flower absolute (LMFAb) on skin-related biological events, especially skin re-epithelization, barrier and moisturizing-related keratinocyte (HaCaT cell) responses. In this study, LMFAb was isolated from LM flowers via solvent extraction and its chemical composition analysis was performed using gas chromatography/mass spectrometry. 5-bromo-2′-deoxyuridine incorporation, Boyden chamber, sprout outgrowth, enzyme-linked immunosorbent, and Western blot assay were used to analyze the biological effects of LMFAb on HaCaT cells (a human epidermal keratinocyte cell line). Twelve components were identified in LMFAb. LMFAb promoted cell proliferation, migration, and sprout outgrowth in HaCaT cells. The absolute enhanced the activations of MAPKs (ERK1/2, JNK, and p38), PI3K and AKT proteins in HaCaT cells and elevated collagen type I and IV levels in HaCaT cell conditioned medium. In addition, LMFAb induced an increase in the expression levels of epidermal barrier proteins (filaggrin and involucrin) in HaCaT cells. Furthermore, LMFAb increased hyaluronan (HA) production and expression of HA synthases (HAS-1, HAS-2, and HAS-3) but decreased HYBID (HA binding protein involved in HA depolymerization) level in HaCaT cells. These findings demonstrate that LMFAb might promote skin re-epithelization, barrier and moisturizing-related beneficial responses in keratinocytes. This study suggests that LMFAb should be considered a potential starting material for the development of cosmetic or pharmaceutical agents that restore the functions of damaged skin.

## 1. Introduction

The skin functions as a barrier that protects the human body from harmful external factors and prevents excessive water loss [1].

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https://doi.org/10.1016/j.heliyon.2024.e24434
Received 23 August 2023; Received in revised form 8 January 2024; Accepted 9 January 2024
Available online 10 January 2024

When skin is injured, a four-step wound-healing process (hemostasis, inflammation, proliferation, and remodeling) is initiated to restore its integrity and normal functioning [2]. These wound-healing steps generally overlap and involve cell migration and proliferation, angiogenesis, re-epithelization, and matrix synthesis, which are regulated by complex cellular, biochemical, and molecular interactions [3]. The skin is composed of epidermis and dermis. The epidermis is the uppermost layer comprised mainly of keratinocytes [4]. Skin re-epithelization is a process that resurfaces wounds with epithelium and involves the migration, proliferation, and differentiation of keratinocytes, and thus keratinocyte migration and proliferation are key re-epithelialization events [3,5,6]. Keratinocyte migratory and proliferative responses are regulated by signaling molecules mediated by interplay between various participants such as extracellular matrix (ECM) (e.g., collagens), growth factors, cells, and chemokines [6].

The stratum corneum (the outermost layer of the epidermis) plays a pivotal role in maintaining the structural integrity of epidermis, which is essential for skin barrier function, and is derived from the differentiation and death of keratinocytes [7]. Filaggrin (FLG), loricrin (LOR), and involucrin (IVL) are key epidermal barrier proteins and the major structural proteins of the stratum corneum and are synthesized by keratinocytes [1,8]. FLG is a protein derived from the dephosphorylation of profilaggrin and can aggregate keratin filaments. In addition, FLG is associated with skin water retention and is a natural moisturizing factor [7,9]. LOR is a structural and reinforcement protein in the stratum corneum and accounts for 70–85% of its total protein mass [7]. IVL is an assembly protein that crosslinks with other structural proteins during stratum corneum formation and serves as a scaffold for the crosslinking of other proteins [8]. Therefore, FLG, LOR, and IVL are essential for the normal barrier function of the epidermis [7,9].

Normal moisture levels in the stratum corneum play a pivotal role in maintaining normal skin barrier function and homeostasis [10]. Hyaluronan (HA, also known as hyaluronic acid) maintains skin moisture levels, especially in the stratum corneum [10]. HA is a major glycosaminoglycan component of ECM and a highly hydrophilic linear carbohydrate polysaccharide that binds with water to form a viscous substance that moisturizes, hydrates, and confers elasticity to skin [11,12]. HA can be synthesized by keratinocytes, fibroblasts, or chondrocytes under the regulation of hyaluronic acid synthase (HAS), which has three isoforms, HAS-1, HAS-2, and HAS-3 [12,13]. HA degradation in skin is mediated by hyaluronan-binding protein involved in hyaluronan depolymerization (HYBID), CD44 (a HA receptor), and hyaluronidases (HYAL2 or HYAL1) [14].

*Lespedeza maximowiczii* (LM) is a plant belonging to the Leguminosae family and Lespedeza genus and is indigenous to Korea and parts of Japan [15,16]. Plants in the genus Lespedeza have been utilized as traditional herbal medicine to treat conditions such as nephritis, azotemia, diuresis, inflammation, hyperpigmentation, and energy depletion [17–19] and have been reported in the scientific literature to have antioxidant, antidiabetic, anti-melanogenic, and bacterial neuraminidase inhibitory effects [18–21]. Furthermore, LM has been reported to have tyrosinase inhibitory and estrogenic activities [19,22]. Many studies have been conducted to promote biological activities beneficial to the skin using various natural materials [23–25]. However, no study has investigated the effects of LM on skin-related biological activities. Thus, in the present study, we aimed to investigate whether the LM flowers-extracted absolute (LMFAb) influences functional skin restoration-related biological events such as skin re-epithelization, skin barrier formation, and skin moisturizing-related responses in human keratinocytes (HaCaT cells). Furthermore, we analyzed the compounds of LMFAb using gas chromatography-mass spectrometry (GC/MS).

## 2. Materials and Methods

### 2.1. Materials

Dulbecco's modified Eagle's medium (DMEM) and phosphate buffered saline (PBS) were purchased from Welgene (Daegu, Korea), and fetal bovine serum (FBS), and trypsin-ethylenediamine tetra-acetic acid (EDTA) were obtained from Gibco BRL (Gaithersburg, MD, USA). Recombinant human epidermal growth factor (rhEGF; purity >97 %) and recombinant human keratinocyte growth factor (rhKGF) were purchased from R&D Systems (Minneapolis, MN, USA). Bovine serum albumin (BSA) was obtained from GenDEPOT (Katy, TX, USA) and dimethyl sulfoxide (DMSO) from MilliporeSigma (St. Louis, MO, USA). The EZ-CyTox assay kit was purchased from DoGen (Seoul, Republic of Korea), and type I collagen from BD Bioscience (Franklin Lakes, NJ, USA). The antibodies used were anti-serine/threonine kinase-protein kinase (AKT), anti-phospho AKT, anti-extracellular signal-regulated kinase (ERK)1/2, anti-phospho ERK 1/2, anti-p38 mitogen-activated protein kinase (MAPK), anti-phospho p38 MAPK, anti-c-Jun N-terminal kinase (JNK), anti-phospho JNK, anti-phosphatidylinositol 3' -kinase (PI3K), anti-phospho PI3K, anti-rabbit immunoglobulin (Ig) G, anti-mouse IgG (all from Cell Signaling, Beverly, MA, USA), anti-HAS-1, anti-HAS-2, and anti-HAS-3 (Novus Biologicals, Littleton, CO, USA), monoclonal anti-type I and IV collagen, polyclonal anti-type I and IV collagen (Invitrogen, Carlsbad, CA, USA), anti-FLG, anti-LOR, anti-IVL (Abcam, Cambridge, UK), HYBID (Santa Cruz Biotechnology, Dallas, TX, USA), and β-actin (MilliporeSigma).

*2.2. Extraction of LMFAb* LM flowers were obtained from a field near a practice farm managed by the Cosmetic Science Division of Hoseo University (Asan, Republic of Korea) (36°44′13.3″N 127°04′42.2E; September 12, 2018) and identified by Dr. Jong-Cheol Yang of the Korea National Arboretum (Republic of Korea). A voucher specimen (No. LMC-0001) was deposited at the Herbarium of the College of Life and Health Science (Hoseo University, Republic of Korea). Absolute was extracted from LM flowers by solvent extraction, as previously described [23]. In brief, 1.74 kg LM flowers were submerged in solvent (n-hexane; Samchun, Pyeongtaek, Republic of Korea) at room temperature (RT) for 1 h. The solvent was removed using a rotary evaporator (EYELA, Tokyo, Japan) at 25°C under vacuum to yield a dark yellow waxy residue (concrete). This residue was dissolved in ethanol, left at −20°C overnight, and filtered through a sintered funnel. The ethanol was then removed by evaporation at 35°C to leave a light yellow anhydrous wax (absolute) (LMFAb; 1.27 g, yield 0.073 % w/w), which was stored at −80°C until required.

### 2.2. Analysis of the compounds in LMFAb

LMFAb was analyzed at the National Instrumentation Center for Environmental Management (NICEM, Seoul National University, Seoul, Korea) by GC/MS. GC/MS data were acquired using a TRACE 1310 GC unit attached to an ISQ LT single quadrupole mass spectrometer (Thermo Scientific, Waltham, MA, USA) as previously described [23]. In brief, the derivatized LMFAb was separated on a J&W VF-5ms capillary column (60 m × 0.25 mm, 0.25 μm; Agilent Technologies, Santa Clara, CA, USA) at a constant flow rate of 1 mL/min using the following procedure; 50°C for 5 min, 50°C–65°C at 10°C/min, 65°C–210°C at 5°C/min, 210°C–310°C at 20°C/min, and held at 310°C for 10 min. The mass range scanned was 35–650 *m/z*, and the data acquisition rate was 0.2 scans/s. The transfer line and ion source temperatures were 300°C and 270°C, respectively. Compounds were identified by comparing their spectra and retention indices (RIs) with reference data extracted from the NIST/NIH/EPA mass spectral library (NIST 11, version 2.0 g) and those of commercially available standards. A standard solution of n-alkanes ($C_9$-$C_{30}$) was used to determine the RIs of each identified compound. In addition, compounds were further identified by comparison with the retention time and spectra of commercially available standards.

### 2.3. Cell culture

HaCaT cell (a human keratinocyte cell line HaCaT) was supplied by Daegu Gyeongbuk Institute for Oriental Medicine Industry (Gyeongsan City, Republic of Korea). HaCaT cells were maintained in DMEM supplemented with 10 % FBS and 1 % penicillin/streptomycin (Hyclone, Logan, UT, USA). Cells were cultured in a humidified atmosphere of 95 % air/5 % $CO_2$ at 37°C and were cultured until 70–80 % confluent for experiments.

### 2.4. Cell viability assay

HaCaT cell viabilities were measured by a water-soluble tetrazolium salt assay using the EZ-CyTox assay kit, as previously described by Kim et al. [23]. Briefly, HaCaT cells ($3 \times 10^3$ cells/well) were seeded into 96-well cell culture plates and treated with different concentrations of LMF absolute for 48 h in a humidified atmosphere of 95 % air/5 % $CO_2$. Cells were then incubated with EZ-CyTox reagent (10 μL/well) for 30 min under the same conditions, and viabilities were assessed at 450 nm using a Synergy 2 multi-well plate reader (Bio-Tek Instruments, Winooski, VT, USA). Cell viabilities were expressed as percentages of untreated controls. The results obtained were used to set concentration ranges for other experiments.

### 2.5. Cell proliferation assay

HaCaT cell proliferation was assessed using a BrdU (5-bromo-2′-deoxyuridine) incorporation assay (Roche, Indianapolis, Indiana, USA). Cells ($3 \times 10^3$ cells/well) were seeded in a 96-well plate, treated with different concentrations of LMFAb in DMEM containing 0.5 % DMSO or the positive control 50 ng/mL rhEGF for 36 h, and then BrdU-labeling solution (10 μM) was added and incubated for 12 h at 37°C to denature DNA. Cells were then treated with peroxidase-labeled anti-BrdU monoclonal antibody and incubated for 90 min at RT. Detection of BrdU antibody complexes was performed using a luminometer (Synergy 2, Bio-Tek Instruments, Winooski, VT, USA), and cell proliferations was assessed by expressing luminesce intensities as percentages of untreated controls.

### 2.6. Cell migration assay

HaCaT cell migration was measured by Boyden chamber assay (Neuro Probe 48-well Micro Chemotaxis Chamber, Neuro Probe Inc., Gaithersburg, MD, USA), as previously described [23]. Briefly, lower chambers were loaded with DMEM medium containing 0.1 % BSA and various concentrations of LMFAb or the positive control rhEGF (2 ng/mL). Membranes coated with type I collagen were then laid over medium in lower chambers. Cells ($5 \times 10^4$ cells/well) were loaded into upper chambers in DMEM containing 0.1 % BSA. Chambers were then assembled and incubated for 210 min at 37°C. Membranes were then fixed and stained using Diff-Quick (Baxter Healthcare, Miami, FL, USA), and cells that migrated onto lower membrane surfaces were counted using an optical microscope ( × 200). Cell migration levels were calculated by expressing migrated cell numbers as percentages of untreated controls.

### 2.7. Sprouting outgrowth assay

Collagen sprout outgrowth assays were performed to assess cell migration and proliferation, as previously described [23]. Briefly, HaCaT cells ($2.5 \times 10^7$ cell/mL) were mixed with type I collagen, 10 × DMEM, and 1 N NaOH (pH 7.2) and spotted ($1.25 \times 10^5$ cells/5 μL) in the wells of a 24-well culture plate. After drying, spots were treated with different concentrations of LMFAb or the positive control 50 ng/mL rhEGF for 48 h at 37°C, fixed, and stained using Diff-Quick. Spots, including sprouts, were visualized and photographed under an optical microscope (at × 100), and sprout lengths were analyzed using Scion Image software (Frederick, MD, USA). Results are expressed as sprout lengths expressed as percentages of those of untreated controls.

### 2.8. Collagen synthesis analysis

Collagen synthesis analysis was performed using an enzyme-linked immunosorbent assay (ELISA), as previously described [23].

Briefly, HaCaT cells were seeded in 100-mm cell culture dishes at $5 \times 10^5$ cells/dish, incubated with the different concentrations of LMFAb for 48 h at 37°C, and centrifuged sequentially for 10 min at 500×*g*, 800×*g*, and 1000×*g*. Supernatants (conditioned media; 100 μL/well) were placed in 96-well microtiter plates pre-coated with type I or IV collagen monoclonal antibody and subjected to incubation with biotin-conjugated polyclonal antibody targeting collagen type I or IV polyclonal antibody (at a dilution of 1:2000 in a solution of 1 % BSA/PBS) for 1 h min at RT. After washing wells with PBS, streptavidin-horseradish peroxidase conjugate (Roche, Indianapolis, IN, USA) (diluted 1:5000 in 1 % BSA/PBS) was added, and plates were incubated at RT for 1 h. After washing with PBS, plates were subjected to incubation with enhanced chemiluminescence (ECL) solution (Thermo Fisher Scientific). Luminescence measurement was performed using a luminometer (Synergy 2, Bio-Tek Instruments), and collagen levels were expressed as percentages of those of untreated controls.

### 2.9. Western blotting

Cells were lysed in radioimmunoprecipitation assay buffer (RIPA buffer; Cell Signaling), and lysates were centrifuged at 17,000×*g* and 4°C for 15 min. Protein concentrations in supernatants were quantified by a DC protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Proteins (30–100 μg/lane) were then separated by 10 % SDS-PAGE and transferred to polyvinylidene fluoride membranes at 4°C. Membranes were blocked in 3% non-fat dry milk for 2 h at RT and washed with 0.05 % Tween-20-containing PBS. The membranes were loaded with each target primary antibody (diluted 1: 1000–5000) and then incubated with horseradish peroxidase-conjugated secondary antibody (diluted 1: 2000–5000) for 1 h at RT. Protein bands were detected using a chemiluminescence substrate and a chemiluminescence imaging system (LuminoGraph, ATTO, Tokyo, Japan). Band quantities were determined using Quantity One software (Bio-Rad, Hercules, CA, USA). For kinases, the expression levels of phosphorylated proteins were normalized versus corresponding total proteins. The percentage of phosphorylated proteins versus total proteins in the untreated state was considered to be 100 %. For FLG, IVL, LOR, HASs, and HYBID, protein levels were normalized versus β-actin. Percentage protein levels were calculated with respect to corresponding untreated controls. β-Actin was used as an internal control.

### 2.10. Hyaluronan release assay

The HaCaT cells ($3 \times 10^5$ cells/well) were added to a 6-well plate for 12 h and starved for more than 6 h in serum-free DMEM to eliminate any FBS effects. Cells were then incubated with different concentrations of LMFAb or the positive control (rhKGF, 20 ng/mL) for 24 h when media were collected and centrifuged sequentially at 500×*g*, 800×*g*, and 1000×*g* for 10 min each. Supernatants (conditioned media; 50 μL/well) were analyzed for HA using an ELISA kit (R&D Systems). HA levels were analyzed using a Synergy 2 multi-well plate reader (Bio-Tek Instruments) at 450 nm.

### 2.11. Statistical analysis

The significances of differences in comparisons between pairs of groups were determined using the Student's *t*-test. Multiple comparisons were performed by One-way analysis of variance (ANOVA) followed by a Dunnett's post hoc test using GraphPad Prism (version 5.0; Graphpad Software, Inc., LaJolla, CA, USA). Results are expressed as means ± standard errors of means (SEMs), and *P* values of $<0.05$ were considered significant.

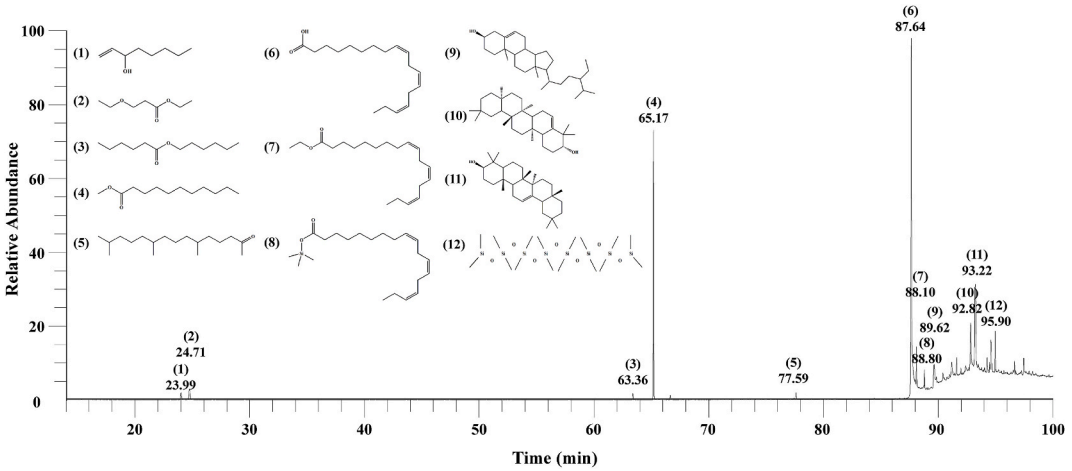


**Fig. 1.** GC/MS total ion chromatogram of *Lespedeza maximowiczii* flower absolute. Numbers bracketed in peaks and numbers below peaks indicate the numbers and retention times of the 12 identified compounds listed in Table 1. Chemical structures of the 12 identified compounds and their number are shown on the upper left of the chromatogram.

## 3. Results

### 3.1. Chemical composition of LMFAb

Compounds in LMFAb were identified by GC/MS, which showed LMFAb contained 12 compounds (Fig. 1 and Table 1). The strongest GC peak was attributed to linolenic acid (39.71 %), and this was followed in descending order by methyl undecanoate (22.96 %), β-amyrin (15.62 %), glutinol (7.14 %), β-sitosterol (4.99 %), ethyl linolenate (2.66 %), ethyl 3-ethoxypropionate (2.10 %), trimethylsilyl (3Z,6Z, 9Z)−3,6,9-octadecatrienoate (1.67 %), 1-octen-3-ol (1.40 %), hexyl hexanoate (0.62 %), 6,10,14-trimethylpentadecan-2-one (0.60 %), and 1H, 15H-hexadecamethyloctasiloxane (0.54 %) (Table 1).

### 3.2. Effects of LMFAb on the proliferation and migration of HaCaT cells

Keratinocyte migration and proliferation play important roles in re-epithelialization of skin [26]. To investigate the effects of LMFAb on keratinocyte migration and proliferation, we first evaluated its cytotoxicity at concentrations of 1–250 μg/mL on HaCaT cells using an EZ-CyTox assay kit. LMFAb did not affect HaCaT cell viability at concentrations of 1, 10, or 150 μg/mL but significantly increased cell viability versus untreated controls at 50and100 μg/mL and significantly decreased cell viability at 200 and 250 μg/mL (Fig. 2A). Thus, these two higher concentrations (200 and 250 μg/mL) were excluded from subsequent experiments. BrdU assays showed treatment with LMFAb (1–150 μg/mL) significantly increased cell proliferation at concentrations from 10 to 100 μg/mL and that this peaked at 50 μg/mL (258.92 ± 10.98 % versus untreated controls) (Fig. 2B).

In addition, migration assays showed LMFAb treatment at 1–150 μg/mL significantly increased HaCaT cell migration at 50 and 100 μg/mL (165.82 ± 7.36 % and 166.84 ± 7.43 %, respectively, versus untreated controls; Fig. 2C and D).

Furthermore, these proliferation and migration results of HaCaT cells were confirmed by sprout outgrowth assays, which simultaneously assess keratinocyte migration and proliferation [23]. LMFAb (1–150 μg/mL) significantly increased HaCaT cell outgrowths compared with untreated control at 50 and 100 μg/mL, peaking at 50 μg/mL (215.86 ± 12.44 % versus untreated controls) (Fig. 3A and B).

### 3.3. Effect of LMFAb on signaling proteins in HaCaT cells

The activations of MAPKs (ERK1/2, JNK, and p38) result in the migration and proliferation of keratinocytes [27,28]. Immunoblotting was used to examine the effect of LMFAb on the MAPK signaling pathways in HaCaT cells. LMFAb (1–150 μg/mL) significantly enhanced the phosphorylation levels of ERK1/2 (Fig. 4A and B), JNK (Fig. 4A and C), and p38 MAPK (Fig. 4A and D) at 50 and 100 μg/mL, and these increases all peaked at 100 μg/mL (222.03 ± 23.96 % of untreated controls [p-ERK1/2, Fig. 4A and B]; 212.94 ± 5.91 % of untreated controls [p-JNK, Fig. 4A and C]; 279.97 ± 21.37 % of untreated controls [p-p38 MAPK, Fig. 4A and D]).

The PI3K/AKT signaling pathway is also associated with keratinocyte migration and proliferation [29], and thus, immunoblotting was used to investigate the effect of LMFAb (1–150 μg/mL) on the activations of PI3K and AKT. LMFAb significantly elevated the phosphorylation levels of PI3K and AKT at 50 and 100 μg/mL, and these increases peaked at 50 μg/mL (222.02 ± 6.94 % [Fig. 4A and E] and 191.43 ± 0.78 % [Fig. 4A and F], respectively, versus untreated controls).

### 3.4. Effect of LMFAb on collagen synthesis in HaCaT cells

Collagen plays a key role in the proliferative and remodeling phases of skin wound healing, and type I and IV collagens contribute to keratinocyte migration and proliferation [3,30,31]. Thus, we evaluated whether LMFAb (1–150 μg/mL) affects collagen synthesis,

**Table 1**
Chemical composition of *Lespedeza maximowiczii* flower absolute.

| No | Component name | RT[a] | RI[b] | | Area (%) | CAS No |
|---|---|---|---|---|---|---|
| | | | Observed | Literature | | |
| 1 | 1-Octen-3-OL | 23.99 | 975 | 975 | 1.40 | 3391-86-4 |
| 2 | Ethyl 3-ethoxypropionate | 24.71 | 981 | 971 | 2.10 | 763-69-9 |
| 3 | Hexyl hexanoate | 63.36 | 1389 | 1387 | 0.62 | 6378-65-0 |
| 4 | Methyl undecanoate | 65.17 | 1427 | 1427 | 22.96 | 1731-86-8 |
| 5 | 6,10,14-Trimethylpentadecan-2-one | 77.59 | 1840 | 1845 | 0.60 | 502-69-2 |
| 6 | Linolenic acid | 87.64 | 2150 | 2199 | 39.71 | 463-40-1 |
| 7 | Ethyl linolenate | 88.10 | 2174 | 2169 | 2.66 | 1191-41-9 |
| 8 | Trimethylsilyl (3Z,6Z,9Z)-3,6,9-octadeca trienoate | 88.80 | 2216 | 2218 | 1.67 | 97,844-13-8 |
| 9 | β-Sitosterol | 89.62 | 2282 | – | 4.99 | 83-46-5 |
| 10 | Glutinol | 92.82 | 2651 | – | 7.14 | 545-24-4 |
| 11 | β-Amyrin | 93.22 | 2701 | – | 15.62 | 559-70-6 |
| 12 | 1H,15H-Hexadecamethyloctasiloxane | 95.90 | 2988 | – | 0.54 | 19,095-24-0 |
| | **Total Identified (%)** | | | | 100.00 | |

[a] RT: retention time.
[b] RI: retention indices. RTs and RIs were determined using a J&W VF-5ms capillary column.

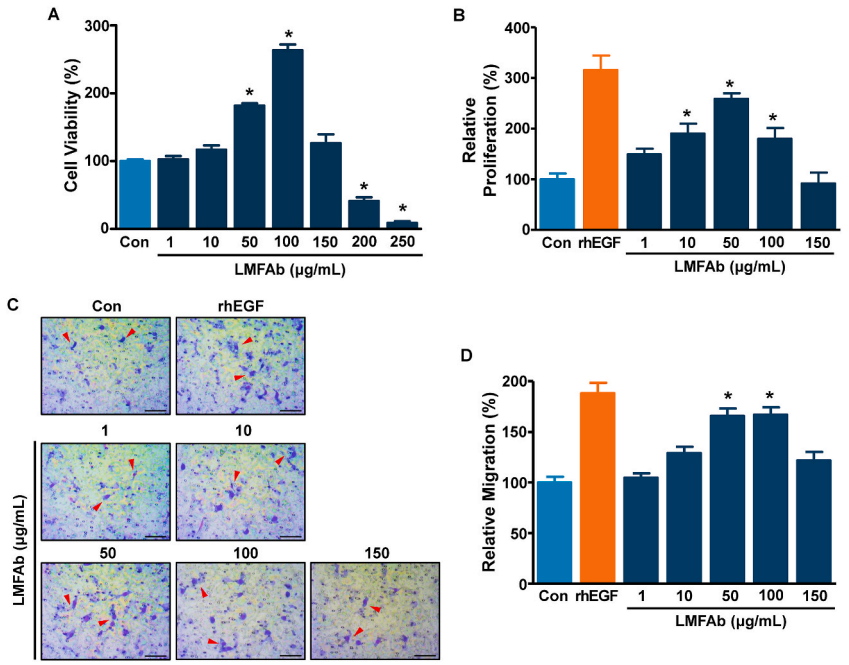


**Fig. 2.** Effects of *Lespedeza maximowiczii* flower absolute on the viability, proliferation, and migration of HaCaT cells. (A) Cell viability. HaCaT cells were treated with *Lespedeza maximowiczii* flower absolute (LMFAb; 1–250 μg/mL) for 48 h. Cell viabilities were determined using the EZ-CytoTox assay kit (n = 5). (B) Cell proliferation. HaCaT cells were incubated with LMFAb (1–150 μg/mL) for 48 h, and cell proliferations were determined using a BrdU incorporation assay as described in Materials and Methods (n = 5). (C-D) Cell migration. HaCaT cells were exposed to LMFAb (1–150 μg/mL) for 210 min, and migration levels were determined using a Boyden chamber assay. (C) Representative images showing the effect of LMFAb on HaCaT cell migration. Violet spots like red arrow head indicate migrated cells. Scale bar = 100 μm. (D) Graphical representation obtained from panel C (n = 4). Recombinant human epidermal growth factor (rhEGF; the positive control) was administered at 50 ng/mL in the proliferation study and at 2 ng/mL in the migration study. Levels in untreated controls (Con) were considered 100 %. Results are presented as means ± SEMs. *$P < 0.05$ vs. untreated cells.

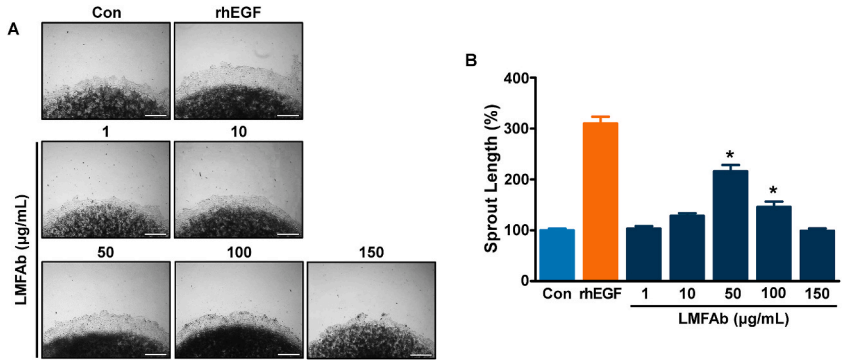


**Fig. 3.** Effect of *Lespedeza maximowiczii* absolute on sprout formation by HaCaT cells. (A) Representative images showing the effect of *Lespedeza maximowiczii* flower absolute (LMFAb) on sprout formation by HaCaT cells. Cells were mixed with collagen and then spotted on a 24-well plate. Cell spots were treated with LMFAb (1–150 μg/mL) for 48 h, stained with Diff-Quick solution, and photographed under an optical microscope. rhEGF (50 ng/mL recombinant human epidermal growth factor): positive control. (B) Graphical representation of the results in panel A. Sprout length level for the untreated control (Con) was taken to be 100% (n = 3). Results are presented as means ± SEMs. Scale bar = 100 μm *$P < 0.05$ vs. untreated cells.

especially collagen type I and IV synthesis by HaCaT cells. LMFAb (1–100 μg/mL) significantly increased the concentrations type I and IV collagens in conditioned media at 50 μg/mL (157.24 ± 5.18 % of untreated controls [type I collagen, Fig. 5A], 171.11 ± 1.22 % of untreated controls [type IV collagen, Fig. 5B]).

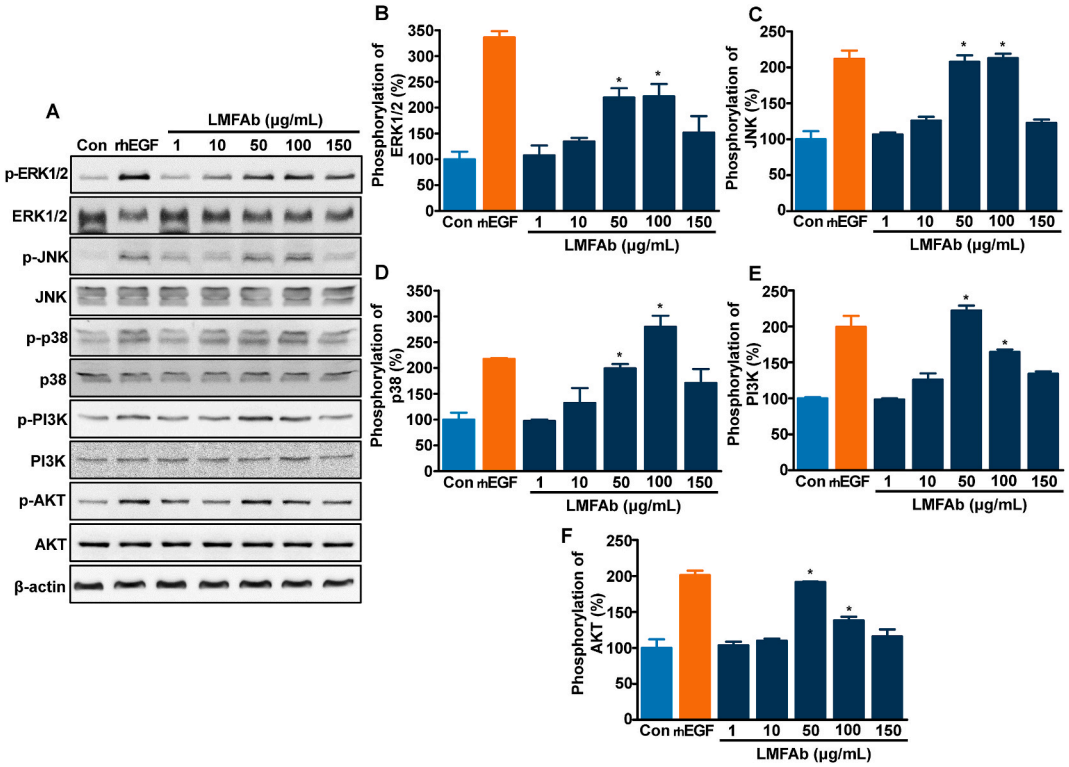


**Fig. 4.** Effect of *Lespedeza maximowiczii* flower absolute on the activations of kinases in HaCaT cells. HaCaT cells were treated with or without LMFAb (1–150 μg/mL) for 10 min. Cell lysates were immunoblotted with each kinase and β-actin antibody. (A) Representative images showing the phosphorylations of MAPKs. Non-adjusted images of Western blotting analysis were shown in Supplemental Fig. S1. (B-F) Graphical representation of the expression levels of phosphorylated ERK1/2 (p-ERK 1/2; B), JNK (p-JNK; C), p38 MAPK (p-p38; D), PI3K (p-PI3K; E), and AKT (p-AKT; F) shown in panel A. The band intensities of phosphorylated proteins were normalized versus corresponding non-phosphorylated proteins. Kinase phosphorylation levels are expressed as percentages of levels in untreated controls (Con). rhEGF (5 ng/mL recombinant human epidermal growth factor): positive control. Results are expressed as means ± SEMs (n = 3/protein). *$P < 0.05$ vs. untreated cells.

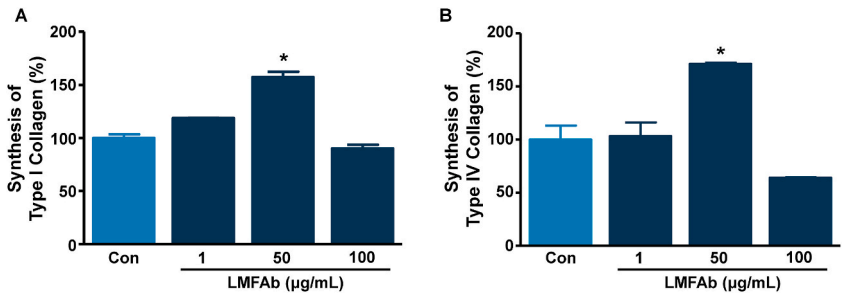


**Fig. 5.** Effects of *Lespedeza maximowiczii* flower absolute on the syntheses of type I and IV collagens. HaCaT cells were incubated in the absence or presence of *Lespedeza maximowiczii* flower absolute (LMFAb; 1–100 μg/mL) for 48 h. Collagen contents in conditioned media were analyzed by sandwich ELISA using anti-type I (A) or anti-type IV collagen antibody (B). The level of each collagen in conditioned media is expressed as percentages of those in untreated controls (Con). Results are expressed as means ± SEMs (n = 3/collagen type). *$P < 0.05$ vs. untreated cells.

### 3.5. Effect of LMFAb on skin barrier proteins in human keratinocytes

FLG, IVL, and LOR are proteins involved in skin barrier formation, and their loss is closely related to skin barrier dysfunction [32]. Thus, immunoblotting was used to investigate whether LMFAb affects their expressions in HaCaT cells. Treatment with LMFAb (1–150 μg/mL) significantly upregulated the levels of FLG (Fig. 6A and B) and IVL (Fig. 6A and C) at 50 and 100 μg/mL, and these upregulations peaked at 50 μg/mL (218.84 ± 19.59% of untreated controls [FLG, Fig. 6A and B]; 182.45 ± 9.08% of untreated controls [IVL, Fig. 6A and C]). However, LMFAb (1–150 μg/mL) treatment did not affect LOR levels (Fig. 6A and D).

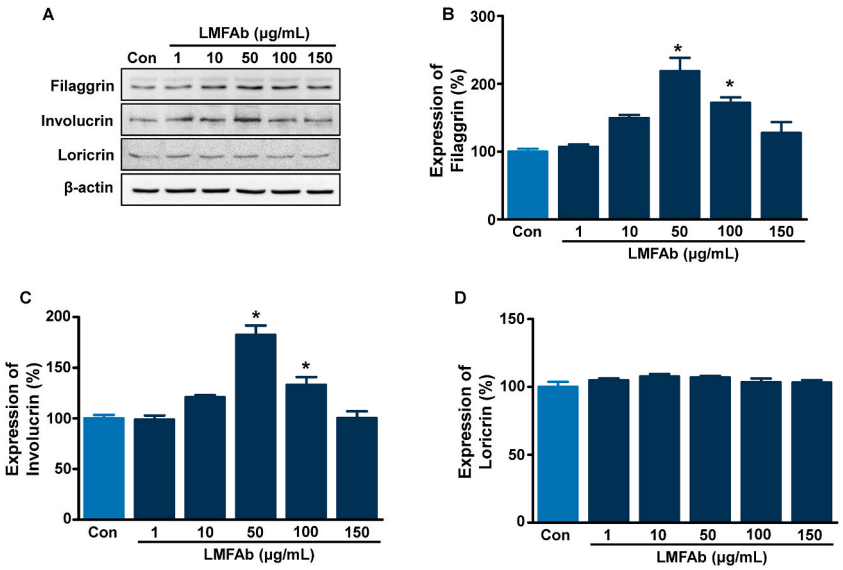


**Fig. 6.** Effects of *Lespedeza maximowiczii* flower absolute on the expressions of skin barrier-related proteins in HaCaT cells. HaCaT cells were cultured for 12 h in DMEM in the presence or absence of *Lespedeza maximowiczii* flower absolute (LMFAb) (1–150 μg/mL). Cell lysates were immunoblotted with the indicated antibodies. (A) Representative images. Non-adjusted images of Western blotting analysis were shown in Supplemental Fig. S2. (B-D) Graphical representation of the results in panel A. The band intensities of expressed proteins were normalized versus β-actin. Protein expression levels are expressed as percentages of those of untreated controls (Con). Results are expressed as means ± SEMs (n = 3/protein). *$P < 0.05$ vs. untreated cells.

### 3.6. Effect of LMFAb on hyaluronan production in HaCaT cells

HA is responsible for maintaining skin moisture levels and is synthesized by HAS (HAS-1, -2, -3) in the skin [12]. Initially, we examined the effect of LMFAb on HAS expressions in keratinocytes. Treatment of HaCaT cells with LMFAb (1–150 μg/mL) significantly

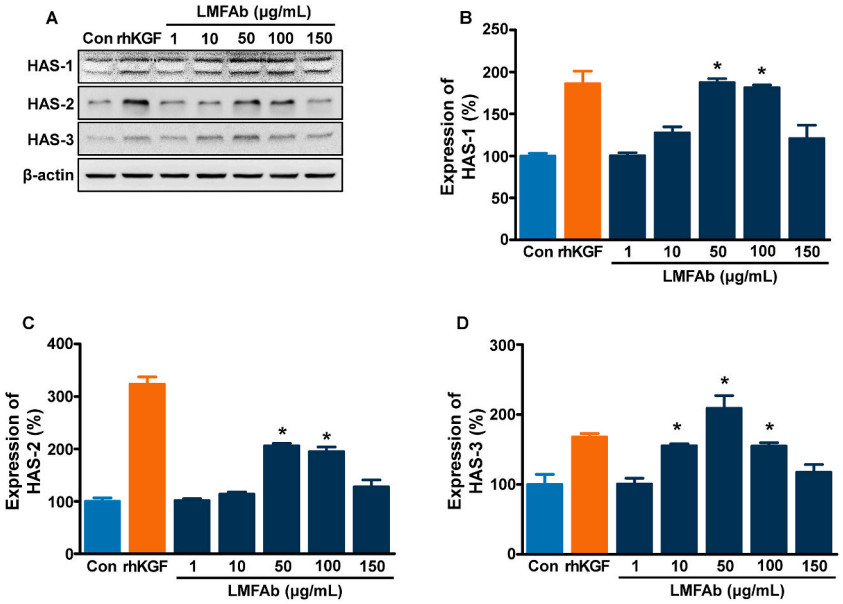


**Fig. 7.** Effects of *Lespedeza maximowiczii* flower absolute on the expressions of hyaluronan synthases in HaCaT cells. HaCaT cells were incubated in the absence or presence of *Lespedeza maximowiczii* flower absolute (LMFAb; 1–150 μg/mL) for 24 h. Cell lysates were western blotted for hyaluronan synthases (HAS-1, HAS-2, and HAS-3) or β-actin. (A) Representative HAS expression images. Non-adjusted images of Western blotting analysis were shown in Supplemental Fig. S3. (B-D) Graphical representation of the results in panel A. Recombinant human keratinocyte growth factor (rhKGF; 20 ng/mL) was used as the positive control. Protein expression levels were normalized versus β-actin. Protein expression levels are expressed as percentages of untreated controls (Con). Results are shown as means ± SEMs (n = 3/protein). *$P < 0.05$ vs. untreated cells.

upregulated the expressions of HAS-1 and -2 in HaCaT cells at 50 and 100 μg/mL, and these increases peaked at 50 μg/mL (187.51 ± 4.60% of untreated controls [HAS-1, Fig. 7A and B]; 205.90 ± 4.60% of untreated controls [HAS-2, Fig. 7A and C]). LMFAb (1–150 μg/mL) also significantly enhanced HAS-3 expression at 10, 50, and 100 μg/mL, and this response peaked at 50 μg/mL (208.93 ± 18.16 % of untreated controls, Fig. 7A and D).

In addition, we tested the effect of LMFAb on HYBID, which participates in HA degradation [14]. LMFAb (1–150 μg/mL) concentration-dependently and significantly attenuated HYBID levels in HaCaT cells at 50–150 μg/mL, and this effect peaked at 150 μg/mL (9.00 ± 0.96 % of untreated controls, Fig. 8A). Finally, an evaluation of the effect of LMFAb on HA production by HaCaT cells showed that LMFAb (1–150 μg/mL) significantly increased HA levels in HaCaT cell condition media at 10–150 μg/mL, and these levels were similar for LMFAb at concentrations from 50 to 150 μg/mL (Fig. 8B).

## 4. Discussion

Delayed or abnormal repair of skin wounds can cause breakdown of the barrier function of skin and lead to infection or even death [33,34]. Therefore, skin wounds must be quickly repaired to restore normal skin functions. Many studies on wound repair and epidermal barrier function have recently described novel therapeutic strategies based on plant-derived extracts [33]. Plants and plant extracts are known to have various biological activities and fewer and less serious side effects than synthetic preparations [24,35], and thus, offer a potential means of safe and effective wound repair and restoring epidermal integrity. In the present study, we found that LMFAb promoted HaCaT cell at concentrations of 10–100 μg/mL and at concentrations of 50 and 100 μg/mL. We also observed that LMFAb did not stimulate proliferation and migration of HaCaT cells at a high concentration (150 μg/mL), which did not negatively affect cell viability. These findings were supported by the results of collagen sprout outgrowth assays, which was used for simultaneous analysis of the migration and proliferation responses in keratinocytes [23]. Our migratory and proliferative results suggest that LMFAb may have the ability to promote proliferation and migration of keratinocytes. Keratinocytes are the main cellular constituents of epidermis [4], and thus, keratinocyte proliferation and migration are essential for re-epithelialization, which is a key event during the proliferation phase of skin repair [3,6]. In addition, GC/MS analysis identified 12 compounds in LMFAb. In particular, β-sitosterol has been reported to promote keratinocyte proliferation [36], implying a potential contribution of β-sitosterol to LMFAb-promoted HaCaT cell proliferation. Therefore, LMFAb may facilitate re-epithelization of the skin by promoting keratinocyte migration and proliferation. Additional studies are required to determine the effects of LMFAb constituents on HaCaT cell proliferation and migration.

Mitogen-activated protein kinases (MAPKs) are activated through phosphorylation of their serine/threonine residues by diverse stimuli, and these activations result in the regulations of multiple cellular functions, such as differentiation, proliferation, apoptosis, and migration [2]. P38, ERK1/2, and JNK MAPKs are three MAPK subfamilies and have been widely reported to be responsible for signaling pathways related to keratinocyte proliferation and migration [25,37–39]. For example, ERK1/2 activation promoted the proliferation and migration of keratinocytes [37,38], and its inhibition had the opposite effect [27,38]. P38 MAPK activation was shown to induce keratinocyte proliferation and migration, and its inhibition abolished keratinocyte migration and proliferation [38, 40]. JNK activation was found to promote or have no effect on the signal transduction pathway responsible for keratinocyte migration and proliferation [28,39], implying that the JNK signaling pathway is not essential for the migratory and proliferative activities of keratinocytes. In the present study, treatment with LMFAb at 50 and 100 μg/mL enhanced activated ERK1/2, p38 MAPK, and JNK levels in HaCaT cells, indicating that LMFAb promotes HaCaT cell migration and proliferation by activating MAPK-mediated signaling pathways. The activation of PI3K and AKT signals also promotes keratinocyte migration and proliferation [25,40]. PI3K inhibitors attenuated AKT phosphorylation in keratinocytes and inhibited keratinocyte proliferation and migration [29], and reduced AKT phosphorylation inhibited keratinocyte proliferation and migration [29, 41, 41]. Our results showed that LMFAb upregulated the phosphorylations of PI3K and AKT in HaCaT cells, suggesting that the PI3K/AKT signaling pathway mediates the LMFAb-induced migration and proliferation of HaCaT cells. Thus, our observations indicate LMFAb promotes HaCaT cell migration and proliferation via MAPKs and/or the PI3K/AKT signaling pathway.

Collagen is a major protein constituent of ECM, serves as a scaffold in skin tissue, and plays critical roles in many cellular responses, including cellular adhesion, migration, and proliferation [42]. In addition, collagen influences skin physiology, such as skin elasticity and moisture levels [43]. Collagens participate in almost all phases of wound healing, especially during the proliferative phase when they participate in the initiation of keratinocyte motility [6,44]. Thus, collagen reductions or loss can lead to abnormal skin conditions by reducing elasticity, causing skin wrinkling and dryness, and preventing skin wounds from healing properly [44,45]. Collagen type I is the most abundant interstitial collagen, and collagen type IV is the major component of basement membrane and has been reported to promote keratinocyte migration and proliferation [3,30,31,44,46]. Furthermore, various plant extracts associated with wound healing promotion have been reported to induce the production and secretion of these two collagen types by keratinocytes [23,25]. In the present study, we found that LMFAb upregulated the syntheses of collagen type I and IV in keratinocytes, which suggests LMFAb has potential use for the treatment of wounds and moisturizing of the skin.

The skin barrier-related proteins, FLG, IVL, and LOR, are key structural components of the stratum corneum and are synthesized by keratinocytes [7,8]. It has been reported that aberrant expressions of these barrier-related proteins are closely associated with impaired skin barrier function in various skin diseases [8,32]. For example, Dang et al. reported that FLG silencing in keratinocytes resulted in skin barrier impairment [47], and Kawasaki et al. found epidermal barrier function was reduced by FLG deficiency in mice [48]. Also, the upregulations of FLG and LOR expressions were reported to have potential use for barrier repair in atopic skin [49], and the restoration of skin barrier function disrupted by cytokines increased FLG and LOR protein levels in keratinocytes [50]. In addition, recovery of downregulated expressions of FLG, IVL, and LOR in dorsal skin paralleled skin barrier improvements in DNCB-exposed mice [51,52]. In the present study, we found that LMFAb enhanced the expressions of FLG and IVL in HaCaT cells but not LOR

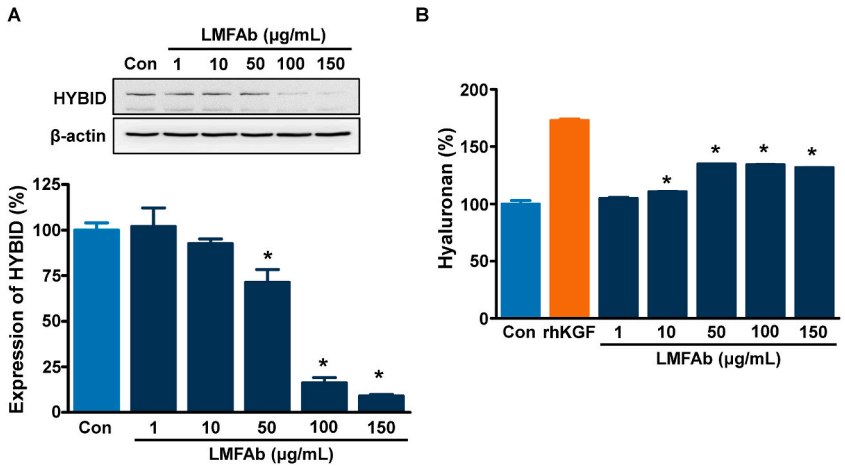


**Fig. 8.** Effects of *Lespedeza maximowiczii* flower absolute on HaCaT cell HYBID expression and hyaluronan release by HaCaT cells. HaCaT cells were cultured in the presence or absence of *Lespedeza maximowiczii* flower absolute (LMFAb; 1–150 μg/mL) for 24 h. Cell lysates were western blotted with HYBID (hyaluronan-binding protein involved in hyaluronan depolymerization) or β-actin antibodies (A). Conditioned media were subjected to ELISA using a Hyaluronan Kit (B). (A) HYBID expression. The upper panel shows representative HYBID expression images. Non-adjusted images of Western blotting analysis were shown in Supplemental Fig. S4. The lower panel is a graphical representation of the results in the upper panel. Protein expression levels were normalized versus β-actin. Protein expression levels are expressed as percentages of untreated controls (Con). Results are presented as means ± SEMs (n = 3). *$P < 0.05$ vs. untreated cells. (B) Hyaluronan release. The levels of hyaluronan release are shown as percentages of levels in the conditioned media of untreated cells (Con) (n = 3). Recombinant human keratinocyte growth factor (rhKGF; 20 ng/mL) was used as the positive control. Data are expressed as means ± SEMs. *$P < 0.05$ vs. untreated cells.

expression. Among the compounds identified in LMFAb, β-sitosterol was previously reported to increase the levels of skin barrier functional proteins, including IVL and FLG, in human keratinocytes [53], and in another study, α-linolenic acid increased FLG expression in keratinocytes [54]. These findings imply that β-sitosterol and α-linolenic acid participate in LMFAb-induced IVL and FLG level increases in HaCaT cells. Therefore, LMFAb may be a potential material for improvement of epidermal barrier function by upregulating the FLG and IVL in keratinocytes.

Stratum corneum moisture levels play a vital role in maintaining skin homeostasis and barrier function [10].

ECM glycosaminoglycan HA is present in the stratum corneum and participates in skin hydration and cell proliferation and migration [11,14]. HA has potent water-binding capacity and thus retains moisture and improves skin elasticity [11,12]. For this reason, HA is considered a key molecule for skin moisture maintenance [12]. HA is synthesized by three HAS isoform enzymes, HAS-1, -2, or -3, in keratinocytes and other cells [12], which implies HA synthesis can be upregulated by enhancing the activities of HASs. HYBID (also known as cell migration-inducing protein (CEMIP) or KIAA1199) participates in HA depolymerization in skin [14]. It was reported that a cytokine mixture inhibited HA depolymerization by down-regulating HYBID expression and increased HA levels in skin cells [55]. Furthermore, vitamin C treatment induced the expression of HYBID mRNA in keratinocytes [56], indicating that HYBID may promote HA degradation in keratinocytes. These observations suggest that HAS and HYBID activities in keratinocytes regulate HA levels. In the present study, LMFAb elevated HA contents and the expressions of HAS-1, -2 and -3 in HaCaT cells, indicating that LMFAb might upregulate HA contents by enhancing the expressions of HAS-1, -2, and-3 in keratinocytes. Moreover, HYBID expression was reduced by LMFAb in HaCaT cells. Therefore, our findings suggest that LMFAb may improves skin hydration by upregulating HAS levels, downregulating HYBID levels, and promoting HA production in keratinocytes.

## 5. Conclusion

In the present study, we identified 12 components in LMFAb and found that LMFAb promoted the migration, proliferation, and sprout outgrowth of HaCaT cells. Moreover, LMFAb enhanced the phosphorylations of PI3K, AKT, and MAPKs (ERK1/2, JNK, and p38 MAPK) and promoted the syntheses of collagens type I and IV and the expressions of skin barrier proteins (FLG and LOR) in HaCaT cells. Furthermore, LMFAb increased the HA and HAS-1, -2, and -3 levels but decreased HYBID levels in HaCaT cells. These findings indicate that LMFAb may have beneficial effects on skin reepithelization, the skin barrier, and skin moisturizing-linked responses in HaCaT cells. Therefore, we suggest LMFAb be viewed as a starting point for the development of cosmetics or treatments for skin conditions.

There are limitations in the present study. LMFAb is a type of essential oil containing many highly volatile components that may be difficult to formulate for application. Moreover, the major bioactive components responsible for the effects of LMFAb on keratinocytes observed above were not clearly identified. Additionally, the *in vitro* effect of LMFAb were not confirmed through *in vivo* testing of LMFAb. These limitations may be essential to be overcome through future related studies in order to utilize LMFAb in the development of cosmetics and pharmaceuticals.

## Data availability statement

Not applicable. Data will be made available on request.

## Funding

This study was supported by a grant from the R&D Program for Forestry Technology of the Korea Forest Service (Korea Forestry Promotion Institute) (Grant No. 2021374B10-2223-BD02) and a Korea Research Fellowship program funded by the Ministry of Science and ICT through the National Research Foundation of Korea (Grant No. 2020H1D3A1A04081360).

## CRediT authorship contribution statement

**Nan Young Kim:** Formal analysis, Data curation. **Kyung Jong Won:** Writing – review & editing, Writing – original draft, Formal analysis. **Do Yoon Kim:** Writing – review & editing, Formal analysis, Data curation. **Da Kyoung Lee:** Writing – review & editing. **Yoon Yi Kim:** Writing – review & editing. **Hwan Myung Lee:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Formal analysis, Data curation, Conceptualization.

## Declaration of competing interest

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

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e24434.

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