

The stimulatory effects of *Stewartia koreana* extract on the proliferation and migration of fibroblasts and the wound healing activity of the extract in mice

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Abstract. *Stewartia koreana* (*S. koreana*) has been used in the treatment of inflammatory diseases, such as acute gastroenteritis and aches, in Korean folk medicine and has been reported to have a number of biological activities, such as anti-inflammatory activity and the promotion of angiogenesis. In this study, we aimed to determine the effects of *S. koreana* extract (SKE) and its components on dermal fibroblast growth and migration, and to investigate the wound healing activity of the extract in mice. *In vitro* experiments revealed that the numbers of SKE-treated cells increased by approximately 2.5- and 3.7-fold with 50 and 100 $\mu\text{g/ml}$ of SKE, respectively. 5-bromo-2'-deoxy-uridine (BrdU) incorporation was also increased in the SKE-treated cells by 2.3-fold. SKE promoted the migration of human skin fibroblasts and, among the isolated compounds, hyperin increased the proliferation and migration of the fibroblasts to almost the same degree as SKE. Western blot analysis demonstrated that SKE stimulated the MEK/ERK1/2 and PI3K/Akt signaling pathways. In *in vivo* experiments, the SKE-treated wound lesions of mice decreased by approximately 7% in diameter after 2 days of treatment with SKE compared with the wound lesions on the 1st day of the experiment. On the 9th day of treatment, the diameter of the lesions was further reduced by approximately 83% in the SKE-treated wound areas compared with the wound areas on the 1st day of treatment. Our results demonstrate that methanol extracts of *S. koreana* leaves promote the proliferation and migration of skin fibroblasts and possess effective wound healing activity through the activation of the

MEK/ERK1/2 and PI3K/Akt signaling pathways. Hyperin was identified as an active compound responsible for the stimulation of fibroblast growth and migration.

Introduction

Wound healing comprises several phases, such as inflammation, granulation, re-epithelization and tissue remodeling (1). The wound healing processes, include cell migration, simultaneous stimulation of new blood vessel formation, the proliferation of cells, such as keratinocytes and fibroblasts, and the production of basement membrane zones and connective tissues. A number of different cell types are recruited to the wounded skin areas during the healing process (2). After an injury to the skin has occurred, inflammatory cells are attracted to the site of infection to remove the damaged tissues, and then fibroblasts and keratinocytes migrate to the wound lesion and proliferate in order to generate new tissue. Fibroblasts, the most common connective tissue cells, are the predominant cell type in the dermis and play a critical role in wound healing. Fibroblasts produce collagen-based extracellular matrix (ECM) and help reapproximate wound edges through their contractile properties. The remodeling phase, which is a period of consolidation and strengthening, promotes a change in the composition of the ECM of fibers and cells (3).

A variety of herbs and plants have been traditionally used for the treatment of various diseases and wounds in folk medicine in many developing countries (4-6). The choice of herbal products for the treatment of wounds and other ailments depends on the tradition and the plant species grown in different regions of the world. In recent years, many efforts have been made worldwide to discover agents from natural resources that can promote wound healing, reduce the cost of treatment, and save patients from other severe complications (7-9). *Stewartia koreana* [*S. koreana*; (Theaceae)], a deciduous tree, is native to Korea, and its tree leaves have been used as a traditional oriental medicine for the prevention and treatment of various diseases. Previous studies have demonstrated that extracts of *S. koreana* exhibit various biological activities, such as anti-inflammatory activity, the inhibition of osteoclast differentiation and angiogenic activity (10-12).

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In the present study, we analyzed the effects of *S. koreana* extract (SKE) and its isolated compounds on the proliferation and migration of normal human dermal fibroblasts (NHDFs) and evaluated its wound healing activity in mice. We found that SKE stimulated the proliferation and migration of fibroblast cells, and, among the isolated compounds, hyperin was the most effective in stimulating the proliferation and migration of NHDFs. Moreover, we demonstrated that SKE stimulated wound healing in the skin of animals. Our results suggest that SKE is very effective in wound healing, and that hyperin may contribute to the strong wound healing activity of SKE by stimulating the proliferation and migration of normal dermal fibroblasts.

Materials and methods

Materials and cell culture. Methanol extracts of *S. koreana* were prepared as previously described (10). Briefly, *S. koreana* leaves were dried and refluxed twice with 80% methanol. The extract was then filtered and centrifuged at 800 x g for 10 min. Methanol in the extract was removed under reduced pressure by rotary evaporation and the water residue was removed by lyophilization. The percentage yield was calculated based on the weight of the dried plant material. The dried powder was dissolved in aliquots of DMSO and diluted to the desired concentrations.

NHDFs were cultured in FGM-2 (Clonetics, Lonza, Walkersville, MD, USA) containing 2% fetal bovine serum (Invitrogen, Carlsbad, CA, USA), 0.1% recombinant human fibroblast growth factor (rhFGF), 0.1% insulin (Clonetics), 5 U/ml of heparin, 100 U/ml of penicillin and 100 µg/ml of streptomycin (Invitrogen) in a humidified incubator at 37°C with 5% CO₂.

Epidermal growth factor (EGF) was obtained from R&D Systems (Minneapolis, MN, USA) and used as the positive control in each experiment. LY294002 (PI3K inhibitor) and PD98059 (MEK inhibitor) were obtained from R&D Systems. Other chemicals were obtained commercially from Sigma-Aldrich (St. Louis, MO, USA).

Isolation and analysis of hyperin in ethyl acetate (EtOAc) extract by high performance liquid chromatography (HPLC). The air-dried leaves of *S. koreana* were extracted and fractionated by successive partitioning as previously described (13). Hyperin was obtained from fractions 15 and 16 by further column chromatography of the pooled fraction. For HPLC, the samples were filtered through a 0.45-µm microspin PVDF filter. The HPLC equipment used was as follows: an Agilent 1200 series system (Agilent Technology Inc., Santa Clara, CA, USA); G1315 DAD detector, G1329A auto sampler, G1322A degasser, G1311 Quat pump; stationary phase Agilent TC-C18 (5 µm, 4.6x250 mm); λ=356 nm; binary gradient (A, water; B, CH₃CN), t_{0min} 50% A → linear gradient to 15% A within 25 min → linear gradient to 5% A/95% B within 14 min → linear gradient to 0% A within 20 min; flow rate 1.0 ml/min; injection volume 1 µl.

Animal experiments. Animal experiments were approved by the Institutional Review Board (IRB), Kyung Hee University, Seoul, Korea. Healthy BALB/c mice (8 weeks, 25-30 x g) were purchased from Central Laboratory Animal Inc. (Seoul, Korea) and divided randomly into 2 groups (control and SKE-treated group) of 6 mice in each. Each mouse was placed in separate

wire-bottom cages in an atmosphere-controlled room (12 h light/dark cycle; temperature, 22±3°C; and 50±10% relative humidity) and maintained on a standard laboratory diet and provided with water.

Wound generation. Mice were anesthetized with 65% N₂, 30% O₂ and 5% isoflurane. Anesthesia was maintained with 2% isoflurane during surgery as previously described (14). Dorsal hair was shaved and wiped with ethanol (70%) prior to generating the wounds. Two horizontal circular wounds (6 mm in diameter) were created on the upper back of each mouse using a biopsy punch instrument. Each wound was topically treated either with EGF (R&D Systems) 200 ng/mouse, PBS or SKE (200 µg/mouse) at the same time each day for 8 days. Images of the wounds were acquired using a digital camera every day immediately before treatment. A small piece of plotting paper (5x5 mm) was used as a standard for the accurate normalization of the wound sizes recorded in the digital images. Wound contraction was calculated using the percentage of wound size as follows: wound area day x was divided by wound area day 0, which was multiplied by 100 (where x = 1, 2, 3, ..., n, the day after injury).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. NHDFs seeded in 96-well plates at a density of 5x10³ cells/well and allowed to attach for 24 h. After discarding the growth medium, the fibroblasts were treated with SKE or various concentrations of phytochemicals isolated from SKE in serum-free medium for 48 h. EGF (R&D systems) was used as a positive control. Following incubation, the cells were treated with 100 µg/ml of MTT for 1 h. The formazan precipitate was dissolved in 200 µl of DMSO and the absorbance at 560 nm was determined spectrophotometrically. The analyses were repeated 3 times and the results are expressed as the means of 3 independent experiments.

BrdU incorporation assay. The 5-bromo-2'-deoxy-uridine (BrdU) incorporation assay was performed using BrdU labeling and a detection kit (Roche, Indianapolis, IN, USA) according to the manufacturer's instructions. In brief, the fibroblasts were seeded in 96-well plates for 24 h. After the removal of the medium, the cells were starved for 6 h and then serum-free medium containing various concentrations of SKE or EGF was added for 24 h. BrdU-integrated DNA was quantified according to the relative luminescence unit (RLU) for each well using a Wallace Victor² 1420 multi-label counter (Perkin-Elmer, Monza, Italy). The analyses were repeated 3 times and the results are expressed as the means of 3 independent experiments.

Scratch wound closure assay. The confluent NHDF monolayers in 6-well plates (SPL Inc. Seoul, Korea) were scratched and wounded using a universal sterile 200-µl pipette tip and then rinsed with PBS. Each well was treated with various concentrations of medium alone (vehicle), SKE or hyperin for 6 h. EGF was added as a positive control. The cells were stained with Diff-Quick (Baxter Healthcare Corp., McGraw Park, IL, USA) and the width of the wound was measured using an Olympus digital camera (Olympus, Tokyo, Japan). The area of the wound was analyzed using the double-blind direct cell

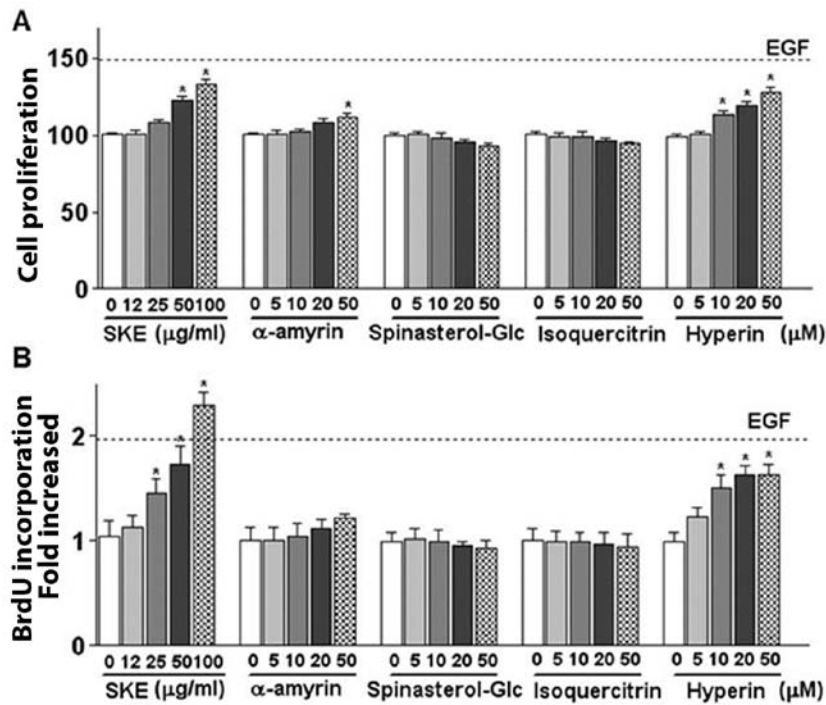


Figure 1. *Stewartia koreana* (*S. koreana*) extract (SKE) and its isolated phytochemicals stimulate the proliferation of human dermal fibroblasts. (A) Proliferation of fibroblast cells was measured by MTT assay as described in Materials and methods. Normal human dermal fibroblasts (NHDFs) were plated in 96-well plates in the presence or absence of SKE and isolated phytochemicals, were allowed to attach for 24 h, and were then treated with the indicated concentrations of each sample for 48 h. Cell growth was measured using a spectrophotometer at 560 nm. (B) The rate of relative DNA synthesis of fibroblasts was measured by BrdU incorporation assay. Fibroblasts were plated in 96-well plates in the presence of the indicated concentrations of SKE and isolated phytochemicals. BrdU-integrated DNA was quantified by measuring the relative luminescence unit (RLU). *p<0.05 vs. cells in medium alone. Data are shown as the means ± SD from 3 independent experiments conducted on triplicate samples.

counting method. Each condition was studied in triplicate wells and each experiment was performed 3 times.

Western blot analysis. The cell extracts were prepared from NHDFs treated with various concentrations of SKE or EGF for 15 min. The cells were lysed in RIPA buffer containing proteinase inhibitors (Roche, Indianapolis, IN, USA) and fractionated by electrophoresis on 12% SDS-PAGE gels and transferred onto a nitrocellulose membrane. Non-specific binding was blocked by soaking the membrane in Tris-buffered saline-Tween-20 (TBS-T) buffer [0.5 mol/l Tris-HCl (pH 7.5), 0.15 mol/l NaCl, and 1 g/l Tween-20] containing 3-5% non-fat dry milk. Phosphorylated (p)-ERK1/2, ERK1/2, p-MEK, MEK, p-PI3K, PI3K, p-Akt and Akt (Cell Signaling, Denver, MA, USA) were utilized as primary antibodies and peroxidase-conjugated antibody was used as a secondary antibody. The membranes were developed with an enhanced chemiluminescence system from GE Healthcare (Piscataway, NJ, USA) and exposed for 30 sec to X-ray film (Fuji Photo Film Co. Ltd, Tokyo, Japan).

Statistical analysis. The data are presented as the means ± SD. Statistical comparisons between groups were performed using one-way ANOVA followed by the Student's t-test. A p-value <0.05 was considered to be statistically significant..

Results

Effects of SKE on the proliferation of human dermal fibroblasts. The proliferation of skin fibroblasts is important in tissue repair

as fibroblasts are involved in migration, proliferation, contraction and collagen production, leading to the deposition of the ECM and the formation of granulation tissue (1-3). Thus, we investigated the effects of SKE on the proliferation of NHDFs at concentrations up to 100 µg/ml using colorimetric MTT assay. As shown in Fig. 1A, SKE significantly enhanced NHDF growth by approximately 30% at a concentration of 100 µg/ml (p<0.05) compared to that of the control untreated cells. In addition, we examined the effects of SKE on DNA synthesis of NHDFs by BrdU incorporation assay. As shown in Fig. 1B, DNA synthesis of NHDFs treated with SKE increased by approximately 2.8-fold compared with the untreated control cells.

Effects of phytochemicals from *S. koreana* leaves on the proliferation of skin fibroblasts. Previous studies have reported on the isolation of various phytochemicals, such as α-amyrin, 3-O-β-D-glucopyranosyl-spinasterol (spinasterol-Glc), and isoquercitrin from methanol extracts by the successive partitioning of the extracts with EtOAc, n-BuOH (13). Thus, in this study, we examined the effects of isolated compounds, such as spinasterol-Glc, α-amyrin and isoquercitrin on the proliferation of skin fibroblasts. Our results revealed that spinasterol-Glc and isoquercitrin did not affect the growth of NHDFs. α-amyrin appeared to slightly stimulate the proliferation of NHDFs (Fig. 1A and B), which may not account for the stimulatory effects of SKE. In an attempt to find a compound that stimulates NHDF proliferation, we further fractionated the subfraction originating from fractions 15 and 16 (13), which resulted in the separation of an additional

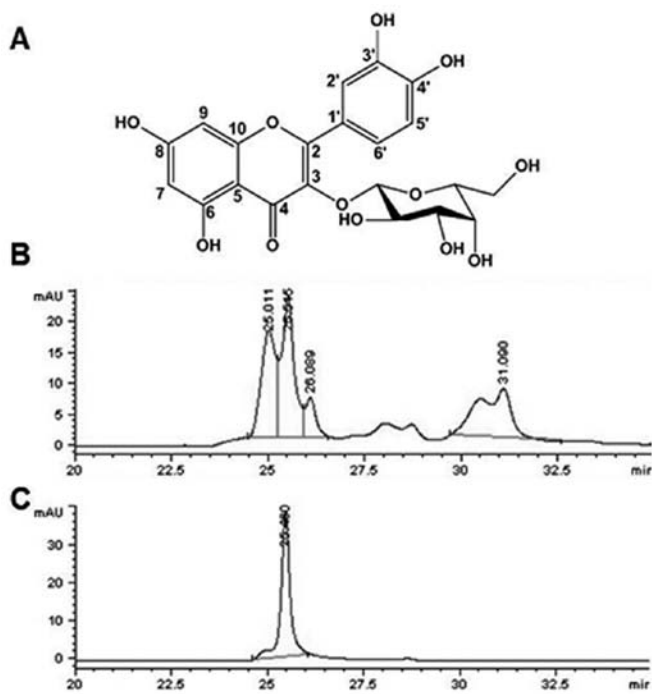


Figure 2. Structure of hyperin and HPLC of EtOAc extract of *Stewartia koreana* (SKE). (A) Structure of hyperin. In HPLC profiles, the peak appearing at 25.5 min was identified as (B) hyperin in EtOAc SKE, and (C) standard hyperin was detected at 25.5 min.

compound. The structure of the compound, which is shown in Fig. 2A, was determined to be hyperin by nuclear magnetic resonance (NMR) spectroscopy (data not shown). HPLC of the EtOAc subfraction revealed that hyperin is a major compound in the fraction (Fig. 2B and C). We evaluated the effects of hyperin on fibroblast proliferation and our results revealed that hyperin strongly stimulated the proliferation of fibroblasts as compared to the control cells (Fig. 1A). Hyperin increased the proliferation of fibroblasts by at least 1.3-fold and stimulated DNA synthesis by approximately 1.8-fold at a concentration of 50 μ M (Fig. 1A and B), which was almost comparable to the effects of SKE.

Effects of SKE and phytochemicals from SKE on the migration of skin fibroblasts. We then investigated the effects of SKE and its phytochemicals on the migration of NHDFs and observed that SKE enhanced the migration of human skin fibroblasts (Fig. 3A and B). When a confluent monolayer of NHDFs was scratched, the migration of cells into the wounded area was significantly increased in the presence of SKE compared to the migration of cells in medium alone 6 h after wounding. SKE at 100 μ g/ml effectively increased the migration of human fibroblasts, which was comparable to the migration induced by EGF. It is therefore plausible that SKE may promote tissue repair not only by increasing proliferation but also enhancing the migration of skin fibroblasts.

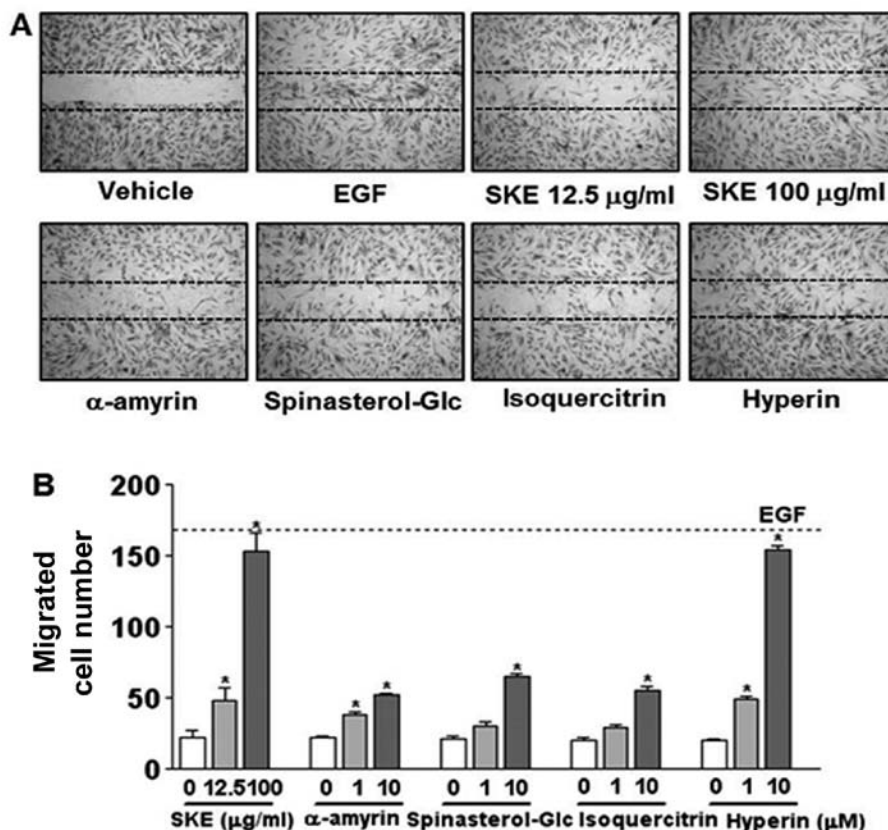


Figure 3. *Stewartia koreana* (*S. koreana*) extract (SKE) and its isolated phytochemicals stimulate the migration of human dermal fibroblasts. Human fibroblasts were seeded in 6-well plates. After 24 h, the cells were washed with PBS. (A) Fibroblasts were treated with SKE and isolated phytochemicals at the indicated concentrations for 6 h and cell migration was detected by wound scratch assays as described in Materials and methods. Representative images of wound scratch assays at the concentration of 12.5, 100 μ g/ml of SKE and 10 μ M of each compound, and a positive control (EGF at 10 nM). (B) The numbers of migrated cells were quantified using double-blind direct cell counting. One representative of 3 experiments is shown. * $p < 0.05$ compared with cells in medium alone.

Table I. Effects of the topical application of methanol extract of *Stewartia koreana* (SKE) on punch wounds on the backs of mice.

| Treatment | Mean lesion diameter (mm ²) | | | | |
|---------------------|---|------------|------------|------------|------------|
| | Day 0 | Day 2 | Day 4 | Day 6 | Day 8 |
| Vehicle (PBS alone) | 24.77±2.15 | 33.15±3.13 | 27.18±3.15 | 20.71±2.42 | 12.11±1.92 |
| SKE (200 µg/mice) | 24.86±2.12 | 22.53±3.17 | 16.25±2.19 | 10.02±1.16 | 4.34±0.39 |
| EGF (200 ng/mice) | 25.01±3.21 | 18.28±2.76 | 14.31±3.01 | 12.04±1.72 | 5.86±0.64 |

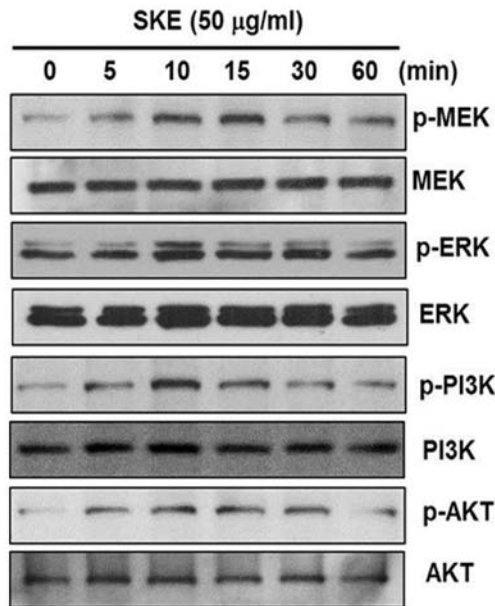


Figure 4. Effects of *Stewartia koreana* extract (SKE) on the activation of ERK and PI3K signaling pathways. Normal human dermal fibroblasts (NHDFs) were treated with 50 µg/ml SKE for the indicated periods of time. The phosphorylation state of MEK, ERK1/2, PI3K and Akt was determined with specific antibodies against the phosphorylated form of each protein. The blots were then stripped and re-probed with the antibodies against corresponding MEK, ERK1/2, PI3K and Akt. As the loading control, the level of total protein was measured.

We further examined the effects of its phytochemicals on the migration of fibroblasts by a wound closure assay. Among the compounds tested, hyperin induced the most prominent stimulatory effect on the migration of NHDFs on the wound areas. When a confluent monolayer was scratched, the migration of the cells into the wounded area was markedly increased in the presence of 10 µM of hyperin compared to the migration of cells in medium alone 6 h after wounding. However, α-amyrin, spinasterol-Glc and isoquercitrin showed only moderate or very weak activities in promoting the migration of fibroblasts compared to hyperin (Fig. 3A and B). Our results demonstrated that hyperin markedly stimulated fibroblast migration. After 6 h, cell migration was enhanced in response to hyperin (10 µM) by 3- to 5-fold over migration in the presence of medium alone (Fig. 3B). Additionally, the levels of NHDF migration induced by 10 µM of hyperin were similar to the those observed for the positive control, EGF (10 ng/ml). Our results revealed that hyperin stimulated the migration and

DNA synthesis in NHDFs, which promoted wound healing in the animal wound model.

SKE induces the proliferation of fibroblasts through the activation of the mitogen-activated protein kinase (MAPK) and Akt signaling pathways. The activation of MAPK and Akt signaling pathways is known to be involved in the migration and proliferation of skin fibroblasts (15). Thus, we investigated the effects of SKE on the phosphorylation of PI3K/Akt and MAPK/ERK1/2. The serum-deprived NHDFs displayed a barely detectable phosphorylation in the absence of SKE (Fig. 4). However, when SKE was added, ERK1/2, MEK, PI3K and Akt were phosphorylated as early as 5 min after the addition of SKE. The phosphorylation reached a maximum level at 10 to 15 min after exposure, and then decreased. These results suggest that SKE enhances the proliferation and migration of fibroblasts through the activation of the ERK1/2 and PI3K signaling pathways.

Inhibition of MEK/ERK1/2 and PI3K/Akt decreases SKE-induced proliferation and migration of fibroblasts. To determine the role of the MEK/ERK1/2 and PI3K/Akt signaling cascades in SKE-induced NHDF proliferation and migration, we analyzed the phosphorylation of signaling molecules in the presence of specific signaling inhibitors, such as PI3K inhibitor (LY294002) and MEK inhibitor (PD98059). The SKE-induced ERK activation was inhibited by PD98059, but not by LY294002, indicating that ERK phosphorylation is mediated by SKE-dependent MEK activation. Akt activation was inhibited by LY294002, but not PD98059, suggesting that SKE activates the PI3K/Akt pathway (Fig. 5A). We then examined the effects of these inhibitors on the SKE-induced fibroblast proliferation and migration. The treatment of NHDFs with PD98059 and Ly294002 resulted in a significant decrease in the proliferation and migration of fibroblasts (Fig. 5B and C).

Effects of SKE on wound healing in mice. Wounds on the skin of mice were treated with SKE, EGF or PBS each day, and then wound repair was observed for 8 days. The square measure of each wound was determined on the basis of the pixel number and was then converted to the percentage of wound size. In the SKE-treated mice, wounds healed from a mean area of 24.86±2.12 mm² to a mean area of 4.34±0.39 mm² (Fig. 6A and Table I). The process of wound healing as a function of time was then depicted as an X-Y plot (Fig. 6B). Eight days after the wound puncture, the wounds were reduced to approximately 83% of the wound sizes on the first day (p<0.05) (Fig. 6B).

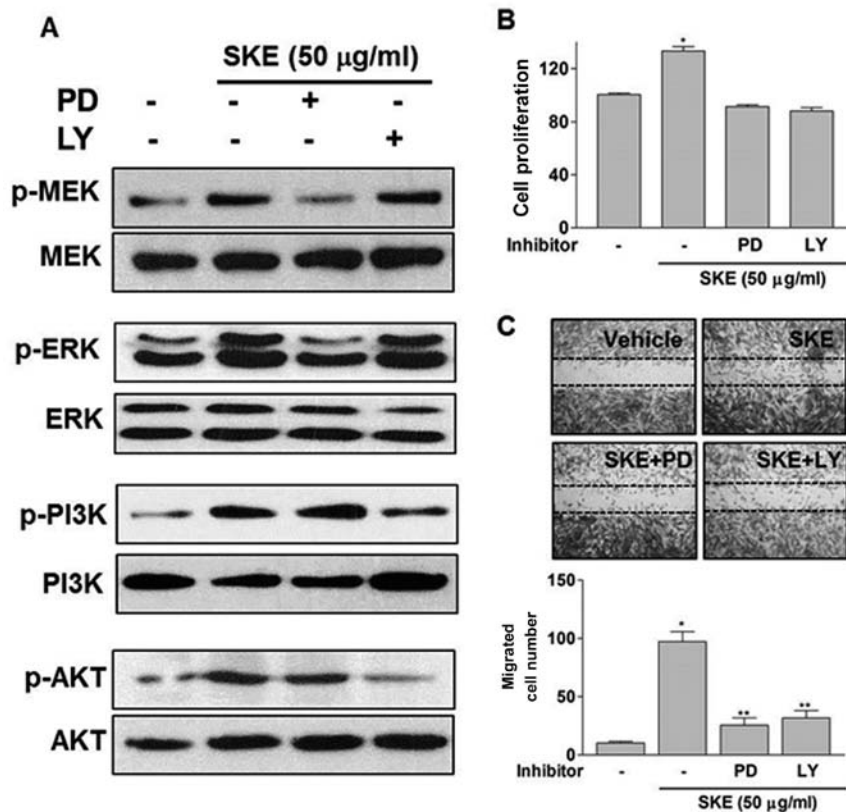


Figure 5. ERK and PI3K signaling pathways are involved in *Stewartia koreana* extract (SKE)-induced fibroblast proliferation and migration. (A) Normal human dermal fibroblasts (NHDFs) were pre-treated for 1 h with inhibitors, such as MEK inhibitor (PD98059) and PI3K inhibitor (LY294002) as indicated and then stimulated with SKE (50 $\mu\text{g/ml}$) for 10 min. Inhibitors were used at the concentration of 10 μM . (B) NHDFs were pre-treated for 1 h with specific inhibitors as indicated, and then treated with 50 $\mu\text{g/ml}$ SKE for 48 h. Cell growth was measured by MTT assay using a spectrophotometer at 560 nm. * $p < 0.05$ compared with negative control values (untreated cells). (C) NHDFs were pre-treated for 1 h with specific inhibitors as indicated. The results of scratch wound assays were photographed (upper panel). The migrated cell numbers were determined by direct cell counting by double blind test (lower panel). Data shown are the means \pm SD ($n=3$). * $p < 0.05$ compared with negative control values (medium alone, vehicle). ** $p < 0.05$ compared with only SKE-treated control values. PD, PD98059; LY, LY294002.

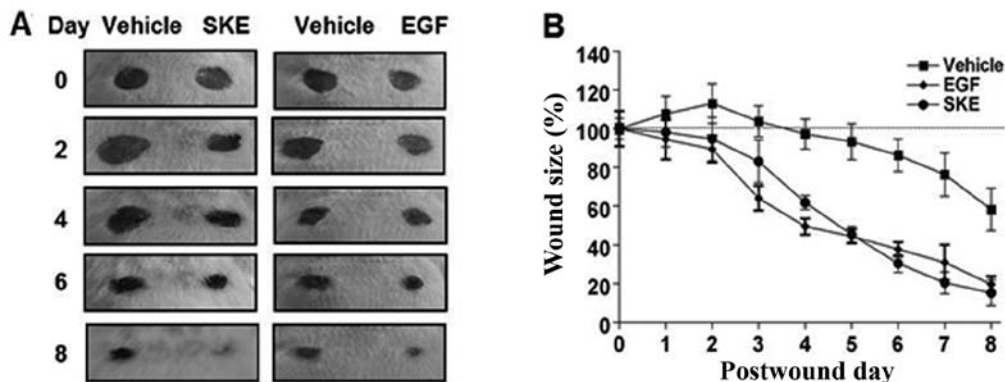


Figure 6. Topical application of *Stewartia koreana* extract (SKE) accelerates wound healing in a punch wound model on the backs of mice. (A) Representative images of mice with baseline wounds. Digital images obtained during the 8 days of the healing process are shown. SKE (200 $\mu\text{g/ml}$) and EGF (200 ng/ml) were applied every 24 h. (B) The square measures of all 12 mice of both the PBS (vehicle)- and SKE-treated groups were precisely measured by pixel and are represented as the percentage of wound size over time. All data are presented as the means \pm SE.

The wound healing activity of SKE was comparable to that of EGF at the concentrations indicated. The wound sizes of SKE-treated skin markedly decreased compared to the wound sizes of PBS-treated skin, indicating that SKE accelerates wound healing *in vivo*.

Our data demonstrated that the healing process of the SKE-treated wounds was accelerated after 3 days, when epithelization and provisional matrix formation begins (1,3). The topical application of SKE enhanced cutaneous wound healing, which appeared to be completed in 8 days. Our results showed

that the original tissue regeneration was much greater in skin wounds treated with SKE than in the control wounds. Moreover, the healing activity of SKE was almost comparable to that of EGF, which is known to accelerate the wound healing process.

Discussion

A number of studies have reported that various plant extracts contain wound healing activities (7-9,16,17). Skin cell behaviors, such as the spreading and migration of fibroblasts, are major determinants of the wound closure rate. In the present study, we demonstrate that SKE stimulates the growth and migration of NHDFs. We also demonstrate that, among the SKE isolated compounds from the methanol extract of SKE, hyperin was the one that most strongly stimulated the growth and migration of fibroblasts. Our results demonstrated that the SKE-induced fibroblast proliferation and migration were mediated by the ERK and PI3K signaling pathways. Furthermore, we showed that the topical application of SKE enhanced the cutaneous wound healing, which appeared to be completed in 8 days. The healing process of the SKE-treated wounds was accelerated after 3 days, when epithelization, angiogenesis and provisional matrix formation began (1). Our results revealed that the original tissue regeneration was much greater in the skin wounds treated with SKE than in the control wounds, and that the healing activity of SKE was comparable to that of EGF.

The stimulatory effects of SKE on fibroblast proliferation and migration seem to be mediated by MEK/ERK, as well as the PI3K/Akt signaling pathways. The phosphorylation of MEK/ERK and PI3K/Akt were barely detectable under the serum-free conditions and the phosphorylation of these molecules was increased after the treatment of fibroblasts with SKE. The inhibition of the ERK pathway by PD98059 and of the PI3K pathway by LY294002 markedly reduced fibroblast proliferation and migration, indicating that the ERK and PI3K pathways are involved in the SKE-induced cell growth and migration. Our results are consistent with those of previous studies, suggesting that the phosphorylation of Akt and ERK1/2 signaling pathways plays an important role in the promotion of proliferation and migration of fibroblasts (15,18,19). The major function of the ERK and PI3K signaling pathways is to stimulate gene transcription involved in proliferation and migration of cells, including fibroblasts (18,19).

We previously isolated a number of compounds from SKE, including α -amyrin, isoquercitrin and spinasterol-Glc. α -amyrin belongs to the triterpenoid family (13), which is known to promote ECM accumulation and have an anti-inflammatory activity (20-22). α -amyrin is one of the most abundant substances in SKE and is presumed to contribute to the wound healing activity of SKE by suppressing inflammation in the wounded areas and enhancing the production of ECM; however, in this study, it did not promote the proliferation and migration of fibroblasts. Spinasterol-Glc has been shown to strongly inhibit the production of nitric oxide (NO) and proinflammatory cytokines in lipopolysaccharide (LPS)-stimulated macrophages (23). Furthermore, spinasterol-Glc promotes the production of procollagen in UVB-irradiated human dermal fibroblast cells (13), and suppresses the production of thymus- and activation-regulated chemokine (TARC/

CCL17) in human keratinocytes (24). Previous studies have reported that isoquercitrin exhibits anti-inflammatory and antioxidant activities (25).

In the present study, we isolated hyperin (also known as hyperoside or quercetin 3-*O*- β -D-galactoside) from SKE and showed that it stimulated the proliferation and migration of dermal fibroblasts in a dose-dependent manner. Hyperin belongs to the flavonoid family, which is mainly found in *Hypericum perforatum* L. (26). It has been previously isolated from various plants and demonstrated to exert multiple bioactivities, including protection from oxidative damage (27), protective effects against neurotoxicity induced by amyloid β -protein (28), and both anti-inflammatory and anti-fungal activities (29-31). The wound healing activities of the *Hypericum perforatum* L. extract have been demonstrated in cultured NIH3T3 fibroblasts and in *in vivo* animal models (32,33). However, to the best of our knowledge, the biological activity of hyperin in stimulating dermal fibroblasts has not been demonstrated to date. Our study demonstrated that hyperin promoted the proliferation and migration of fibroblast, indicating that it can contribute to the wound healing activities of plant extracts, such as *S. koreana*. Taken together, our data demonstrate that *S. koreana* leaves contain various phytochemicals, including hyperin, spinasterol-Glc, α -amyrin and isoquercitrin, which are beneficial for skin wound healing.

In conclusion, this study demonstrates that SKE promotes the proliferation and migration of human fibroblasts *in vitro*, which may contribute to the enhancement of the wound healing process. Consequently, we identified hyperin as an active constituent and a major promoter of fibroblast proliferation and migration. The methanol extracts of leaves from *S. koreana* enhanced skin wound healing in an animal model. Thus, *S. koreana* leaves and their isolated compound, hyperin, may be potentially useful for the treatment of wounds and for the development of agents contributing to the topical treatment of wounds.

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