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Decursin and decursinol angelate improve wound healing by upregulating transcription of genes encoding extracellular matrix remodeling proteins, inflammatory cytokines, and growth factors in human keratinocytes

Jisu Han ^{a,1}, Wook Jin ^{c,d,1}, Ngoc Anh Ho ^{a,1}, Jeongpyo Hong ^b, Yoon Ju Kim ^b,
Yungyeong Shin ^b, Hanki Lee ^{a,*}, Joo-Won Suh ^{a,b,**}

^a Center for Nutraceutical and Pharmaceutical Materials, Myongji University, Yongin, Gyeonggi-do, 17058 Republic of Korea

^b Division of Biosciences and Bioinformatics, College of Natural Science, Myongji University, Yongin, Gyeonggi-do, 17058 Republic of Korea

^c Laboratory of Molecular Disease and Cell Regulation, Department of Molecular Medicine, School of Medicine, Gachon University, Incheon, 21936 Republic of Korea

^d Gachon Medical Research Institute, Gil Medical Center, Incheon, 21565 Republic of Korea

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ABSTRACT

The coumarins decursin and decursinol angelate, which are found in *Angelica gigas* Nakai, have a variety of biological functions. Here, we show that treatment with these compounds improves wound healing by HaCaT human keratinocytes. Wound healing was increased by treatment with up to a threshold concentration of decursin, decursinol angelate, a mixture of both, and a nano-emulsion of these compounds, but inhibited by treatment with higher concentrations. Immunoblotting and fluorescence imaging of cells expressing an epidermal growth factor receptor (EGFR) biosensor demonstrated that these compounds did not stimulate wound healing by inducing EGFR phosphorylation. Rather, transcriptional analysis revealed that decursin and decursinol angelate improved wound healing by upregulating the expression of genes encoding extracellular matrix remodeling proteins, inflammatory cytokines, and growth factors.

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1. Introduction

Coumarins are found in the fruits, roots, stems, and leaves of numerous plants, and are particularly abundant in cassia leaf oil, lavender oil, and cinnamon bark oil. These compounds have been used as anti-coagulants and to treat various clinical conditions including cancer, chronic infections, inflammation, and edema [1]. Coumarins are often classified into four major groups, namely, simple coumarins, furanocoumarins, pyrano-coumarins, and pyrone-substituted coumarins, according to their structures. This structural variation is thought to underlie the diverse biological

functions of coumarins [2].

Decursin and decursinol angelate, two major coumarins in *Angelica gigas* Nakai, can improve blood circulation and have anti-cancer, anti-inflammatory, and anti-oxidation activities, similar to other coumarins found in plants [3–5]. Consequently, these compounds have been used to manufacture therapeutic agents and functional foods [5].

Wound healing is closely related to the production of inflammatory cytokines [6]. In general, wound healing by keratinocytes involves three stages: the inflammation process, tissue formation, and tissue remodeling [6]. Inflammatory cytokines such as interleukin (IL)1, IL6, IL8, and IL10 are produced during the inflammation process. Growth factors such as platelet-derived growth factor, transforming growth factor- β , hepatocyte growth factor (HGF), and epithelial growth factor (EGF) are also synthesized, which play a crucial role in linking the inflammation process with tissue formation [7]. This study investigated the effects of decursin and decursinol angelate on wound healing by HaCaT human

* Corresponding author. Center for Nutraceutical and Pharmaceutical Materials, Myongji University, Yongin, Gyeonggi-do, 17058 Republic of Korea.

** Corresponding author. Division of Biosciences and Bioinformatics, College of Natural Science, Myongji University, Yongin, Gyeonggi-do, 17058 Republic of Korea.

E-mail addresses: hklee95@mju.ac.kr (H. Lee), jwsuh@mju.ac.kr (J.-W. Suh).

¹ These authors contributed equally to this study.

keratinocytes and the transcription of genes related to human wound healing.

2. Materials and methods

2.1. Preparation of decursin and decursinol angelate

Purified decursin and decursinol angelate were purchased from Chengdu Biopurify Phytochemicals (China) and mixed at a ratio of 1.2:1, which is identical to their content ratio in *A. gigas* Nakai. This mixture is hereafter referred to as decursin complexes.

A reversed-phase column (YMC Pack ODS-A, 4.6 $\Phi \times 250$ mm, 5 μ m) was used to determine the purity of decursin and decursinol angelate. The mobile phase comprised 50% acetonitrile and 0.1% formic acid. Chromatography was performed at room temperature with a flow rate of 0.8 ml/min, a wavelength of 329 nm, and an injection volume of 10 μ l.

2.2. Preparation of a nano-emulsion of decursin complexes

A nano-emulsion of decursin complexes was prepared using the oil-in-water method [8]. Briefly, a solution of purified decursin complexes dissolved in 2 ml methylene chloride was added dropwise to 8 ml of 0.2% gelatin dissolved in deionized water and simultaneously homogenized at 9000 rpm for 5 min during dropping the solution of purified decursin complexes. Thereafter, methylene chloride was completely removed by stirring the mixture for 12 h. The size of molecules in the nano-emulsion was determined using a Zetasizer Nano ZS instrument (Malvern). A reversed-phase column (YMC Pack ODS-A, 4.6 $\Phi \times 250$ mm, 5 μ m) was used to analyze the decursin and decursinol angelate contents of the nano-emulsion. The mobile phase comprised 50% acetonitrile and 0.1% formic acid. Chromatography was performed at room temperature with a flow rate of 0.8 ml/min, a wavelength of 329 nm, and an injection volume of 10 μ l.

2.3. Scratch wound healing assay

The scratch wound healing assay was performed as previously described with some modifications [9]. Briefly, HaCaT cells were seeded into a 6-well plate at a density of 5×10^5 cells/well in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μ g/ml streptomycin. Cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO₂. When cells reached 95% confluency, they were scratched with a 200 μ l pipette tip and then treated with various concentrations of decursin, decursinol angelate, decursin complexes, and the nano-emulsion of these complexes. Cells were also treated with 5 nM human EGF (hEGF) as a positive control.

Wound healing was monitored using an inverted microscope (Olympus, Japan) with a 10 $\times$ objective lens over two consecutive days, and quantified using the MRI Wound Healing Tool of ImageJ (http://dev.mri.cnrs.fr/projects/imagej-macros/wiki/Wound_Healing_Tool).

2.4. Analysis of epidermal growth factor receptor (EGFR) phosphorylation

HaCaT cells were cultured in DMEM containing 10% FBS, 100 units/ml penicillin, and 100 μ g/ml streptomycin until they reached 90% confluency, starved overnight in DMEM lacking supplements, and then treated with hEGF or decursin complexes in supplemented medium for 1 day. Thereafter, HaCaT cells were homogenized in RIPA buffer, which contained 100 μ g/ml

phenylmethylsulfonyl fluoride (Sigma, St. Louis, MO), 100 mM sodium orthovanadate (Sigma), and 50 μ l/ml proteinase inhibitor cocktail (Sigma). Western blotting was performed using total cell lysates. The protein content was determined using the Bio-Rad Protein Determination Assay (Bio-Rad, Hercules, CA). Cell lysates were separated by SDS-PAGE and transferred to a PVDF membrane (Bio-Rad). The membranes were incubated with appropriate primary antibodies followed by a horseradish peroxidase (HRP)-conjugated secondary antibody. Bands were visualized by enhanced chemiluminescence (Amersham) and quantified by densitometry (UnScan-It, Silk Scientific, Ore, UT). Antibodies against extracellular signal-regulated kinase 1/2 (ERK1/2), phospho-ERK1/2 (Thr202/Tyr204), phospho-EGFR (Tyr1173), and EGFR were purchased from Abcam. An anti-rabbit HRP-conjugated secondary antibody was purchased from Bio-Rad.

A549 cells expressing an EGFR biosensor were maintained in RPMI 1640 medium supplemented with 10% FBS, 2 mM L-glutamine, and 1 μ g/ml puromycin [10]. To monitor EGFR phosphorylation, these cells were cultured in a glass bottomed dish (Cellvis) until they reached 80% confluency, starved overnight in RPMI 1640 medium lacking supplements, and then treated with hEGF and decursin complexes in supplemented medium. Cells were imaged every 10 min for a total of 1 h using an inverted fluorescence microscope (Nikon) with a 40 $\times$ objective lens (0.6 NA), an excitation wavelength of 490/20 nm (bandwidth: 20 nm, Chroma), and an emission wavelength of 525/36 nm (Chroma) [10]. Images were captured using a EMCCD camera (iXon Ultra, Andor) and Andor imaging software.

2.5. Cell viability assay

The cytotoxic effects of purified decursin, decursinol angelate, and decursin complexes were evaluated using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay (Sigma Chemical Co., St. Louis, MO). HaCaT cells were seeded into 96-well microplates at a density of 1×10^4 cells/well and treated with various concentrations of each compound. After the indicated amount of time, MTT (1 mg/ml) was added, cells were incubated for a further 2 h, and the optical density at 570 nm was measured using a microplate reader (Tecan Austria GmbH, Grödig, Austria). The MTT assay was carried out in triplicate.

2.6. Transcriptional analysis of genes related to human wound healing

Total RNA was isolated from HaCaT cells treated with decursin or decursinol angelate using an RNeasy Mini Kit (Qiagen) and reverse-transcribed using Hexanucleotide Mix (Roche, Mannheim, Germany). Gene expression profiling was performed using the Human Wound Healing RT2 Profiler PCR Array (PAHS-121Z; Qiagen, Valencia, CA) and the 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). Data were analyzed using the PCR Array Data Analysis Web Portal of Qiagen.

3. Results

3.1. Effects of decursin and decursinol angelate on wound healing by HaCaT cells

To determine whether decursin and decursinol angelate improve wound healing, HaCaT human keratinocytes were scratched and then treated with various concentrations of these compounds separately. The scratch was completely closed following treatment with the positive control hEGF for 1 day (Fig. 1). Moreover, the scratch was fully closed upon treatment with

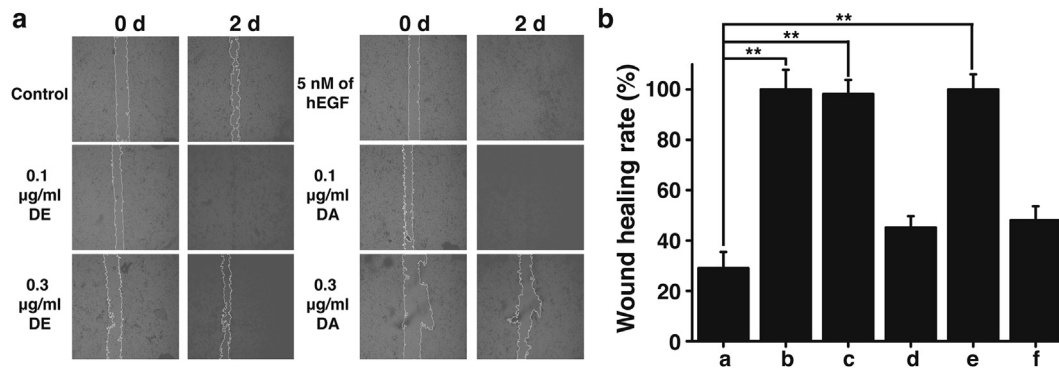


Fig. 1. Scratch wound healing by HaCaT cells treated with decursin and decursinol angelate. a. Comparison of wound healing between HaCaT cells treated with hEGF, decursin, and decursinol angelate. DE and DA indicate decursin and decursinol angelate, respectively. In the case of hEGF, the image was captured after 1 day of treatment. Images were acquired with a 10 × objective lens. b. Quantification of wound closure based on the difference between the gap width at 0 and 2 days. a: No treatment; b: 5 nM hEGF; c: 0.1 µg/ml decursin; d: 0.3 µg/ml decursin; e: 0.1 µg/ml decursinol angelate; and f: 0.3 µg/ml decursinol angelate. The wound healing rate was calculated based on the captured images. ** indicates $P < 0.01$, as assessed using the paired t -test. All error bars indicate standard deviation, unless specified otherwise.

0.1 µg/ml decursin or decursinol angelate for 2 days, whereas treatment with a higher concentration inhibited scratch closure (Fig. 1).

3.2. Effect of decursin complexes on wound healing by HaCaT cells

To determine whether treatment with both compounds had a synergistic effect on wound healing, HaCaT cells were scratched and then treated with various concentrations of decursin complexes, which were prepared by mixing decursin and decursinol angelate at a ratio of 1.2:1 (Supplementary Fig. 1). The scratch was completely closed following treatment with the positive control hEGF for 1 day (Fig. 2). After 2 days, the gap width was reduced by 50% and 72% in cultures treated with 0.1 and 0.5 µg/ml decursin complexes, respectively (Fig. 2). By contrast, treatment with 3.0 and 30 µg/ml decursin complexes inhibited scratch closure (Fig. 2), consistent with the previous experiment. Treatment with decursin complexes did not have a synergistic effect on wound healing.

3.3. Effect of a nano-emulsion of decursin complexes on wound healing by HaCaT cells

We prepared a nano-emulsion of decursin complexes via the oil-in-water method using 2% gelatin solution as a nano-carrier. Most molecules in the nano-emulsion of decursin complexes measured

102.7 nm, and the ratio of decursin:decursinol angelate was 1.2:1 (Supplementary Fig. 2). HaCaT cells were scratched and then treated with various concentrations of this nano-emulsion of decursin complexes. After 2 days, the gap width was reduced by 47% and 73% in cultures treated with 0.01 and 0.05 µg/ml nano-emulsion, respectively (Fig. 3). By contrast, scratch closure was inhibited by treatment with concentrations of this nano-emulsion higher than 0.1 µg/ml. This is consistent with the finding that treatment with 3.0 µg/ml or higher concentrations of decursin complexes perturbed scratch closure (Figs. 1 and 2). These results indicate that treatment with an appropriate concentration of the nano-emulsion of decursin complexes can enhance wound healing by human keratinocytes. The improved permeability of the nano-emulsion explains why it enhanced scratch closure at a lower concentration than decursin complexes.

3.4. Effect of decursin complexes on EGFR signaling in HaCaT cells

To elucidate the mechanism by which decursin complexes improve scratch closure by HaCaT cells, we monitored phosphorylation of EGFR and ERK1/2 because EGFR is a crucial membrane protein related to wound healing by human keratinocytes. Treatment with decursin complexes did not significantly affect phosphorylation of EGFR or ERK1/2 in comparison with hEGF treatment (Fig. 4a). Next, we monitored the direct binding of decursin

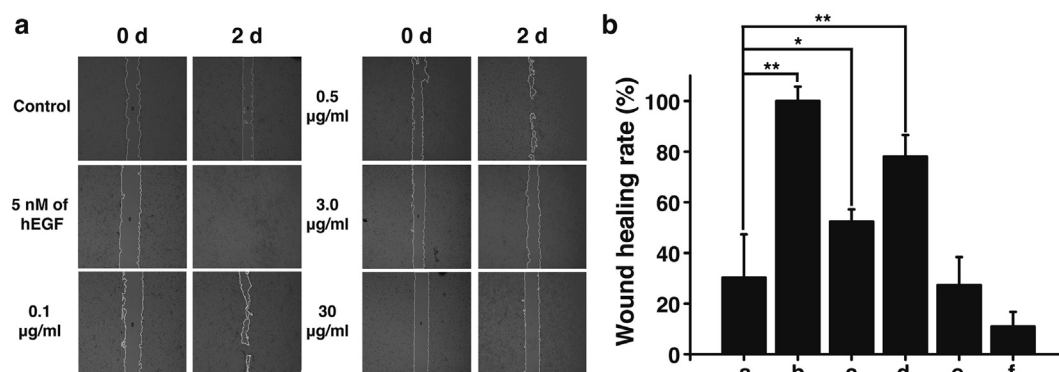


Fig. 2. Scratch wound healing by HaCaT cells treated with decursin complexes. a. Comparison of wound healing between HaCaT cells treated with hEGF and various concentrations of decursin complexes. In the case of hEGF, the image was captured after 1 day of treatment. Images were acquired with a 10 × objective lens. b. Quantification of wound closure based on the difference between the gap width at 0 and 2 days. a: No treatment; b: 5 nM hEGF; c: 0.1 µg/ml decursin complexes; d: 0.5 µg/ml decursin complexes; e: 3 µg/ml decursin complexes; and f: 30 µg/ml decursin complexes. The wound healing rate was calculated based on the captured images. * and ** indicate $P < 0.05$ and $P < 0.01$, respectively, as assessed using the paired t -test. All error bars indicate standard deviation, unless specified otherwise.

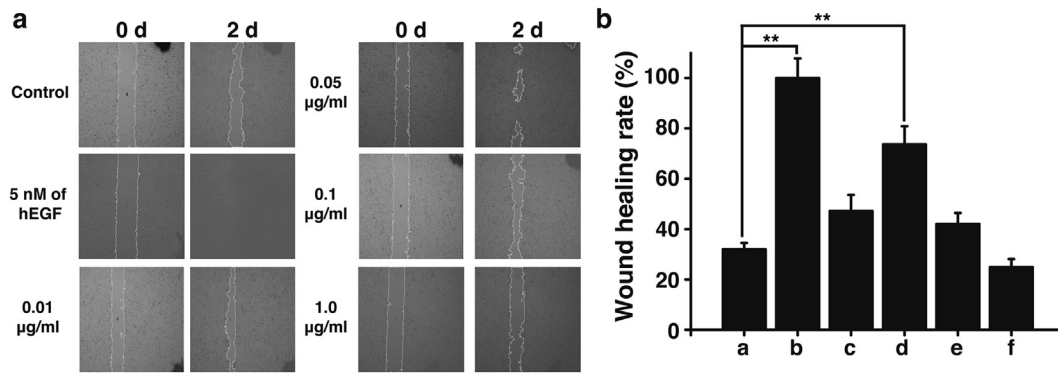


Fig. 3. Scratch wound healing by HaCaT cells treated with a nano-emulsion of decursin complexes. a. Comparison of wound healing between HaCaT cells treated with hEGF and various concentrations of a nano-emulsion of decursin complexes. In the case of hEGF, the image was captured after 1 day of treatment. Images were acquired with a $10\times$ objective lens. b. Quantification of wound closure based on the difference between the gap width at 0 and 2 days. a: 2% Gelatin; b: 5 nM hEGF; c: 0.01 µg/ml nano-emulsion; d: 0.05 µg/ml nano-emulsion; e: 0.1 µg/ml nano-emulsion; and f: 1.0 µg/ml nano-emulsion. The wound healing rate was calculated based on the captured images. ** indicates $P < 0.01$, as assessed using the paired *t*-test. All error bars indicate the standard deviation, unless specified otherwise.

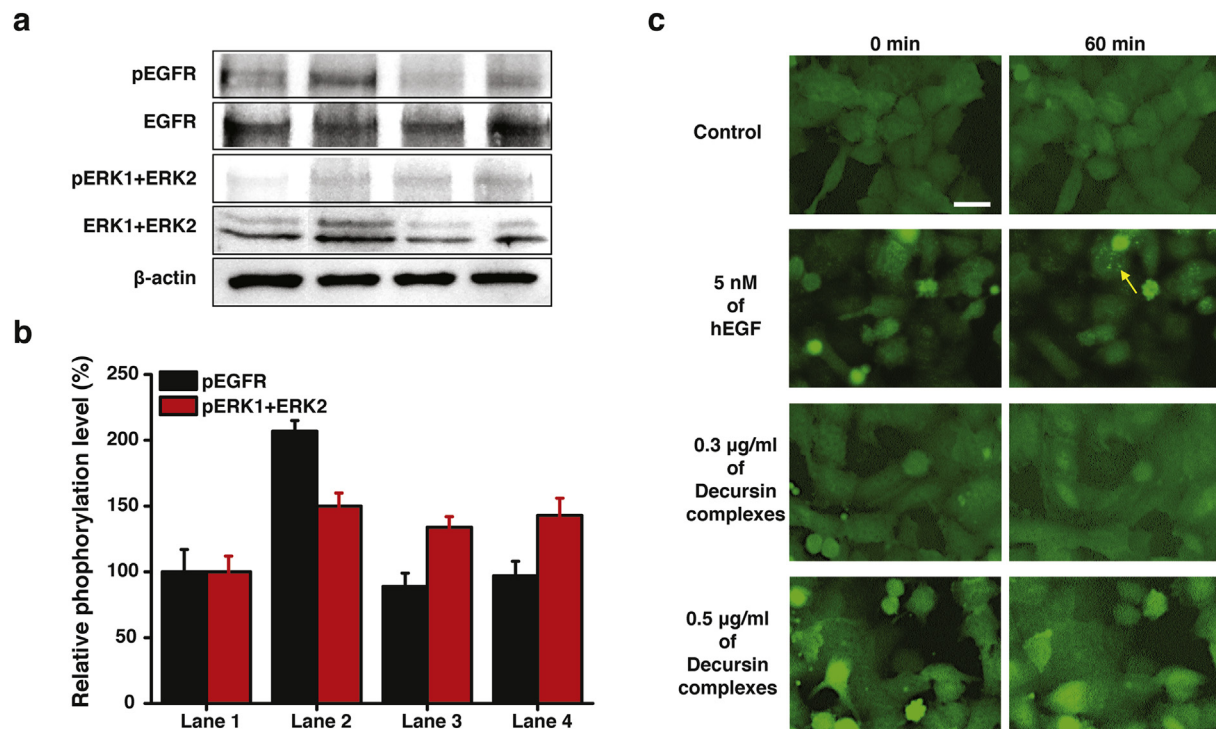


Fig. 4. Phosphorylation of EGFR and ERK1/2 in HaCaT cells treated with decursin complexes. a. Phosphorylation of EGFR and ERK1/2 in HaCaT cells treated with hEGF and decursin complexes. Lane 1: No treatment; lane 2: 5 nM hEGF; lane 3: 0.3 µg/ml decursin complexes; and lane 4: 0.5 µg/ml decursin complexes. b. Quantification of EGFR and ERK1/2 phosphorylation in HaCaT cells treated with hEGF and decursin complexes. The densities of the bands were determined using ImageJ. Black and red bars indicate the levels of phospho-EGFR and phospho-ERK1/2, respectively. c. EGFR phosphorylation in A549 cells expressing an EGFR biosensor and treated with hEGF or decursin complexes. The white bar and yellow arrow indicate 10 µm and the granule by EGFP phosphorylation, respectively.

complexes to EGFR using an EGFR biosensor. This demonstrated that decursin complexes did not bind directly to EGFR (Fig. 4b). Therefore, the wound healing by treatment of decursin complexes at least is not related to EGFR signaling but might be happened by other signaling because the level of ERK phosphorylation by treatment of decursin complexes was similar to the level of ERK phosphorylation by treatment of EGF.

Effects of decursin and decursinol angelate on the transcription of genes related to wound healing in HaCaT cells.

Finally, we analyzed the transcription of genes related to wound healing in HaCaT cells treated with decursin or decursinol angelate

using a Human Wound Healing PCR Array. A total of 22 and 34 genes were upregulated in HaCaT cells treated with decursin and decursinol angelate, respectively, compared with non-treated cells (Fig. 5). CCL7, CSF2, CSF3, CTSG, ITGB3, and SERPINE1 were simultaneously upregulated in cells treated with decursin (Fig. 5a). Meanwhile, 24 genes were upregulated more in cells treated with decursinol angelate than in those treated with decursin (Fig. 5b). Transcription of the other genes was upregulated to a similar degree in cells treated with decursin and those treated with decursinol angelate (Fig. 5). These results indicate that decursin and decursinol angelate, which have similar chemical structures, can

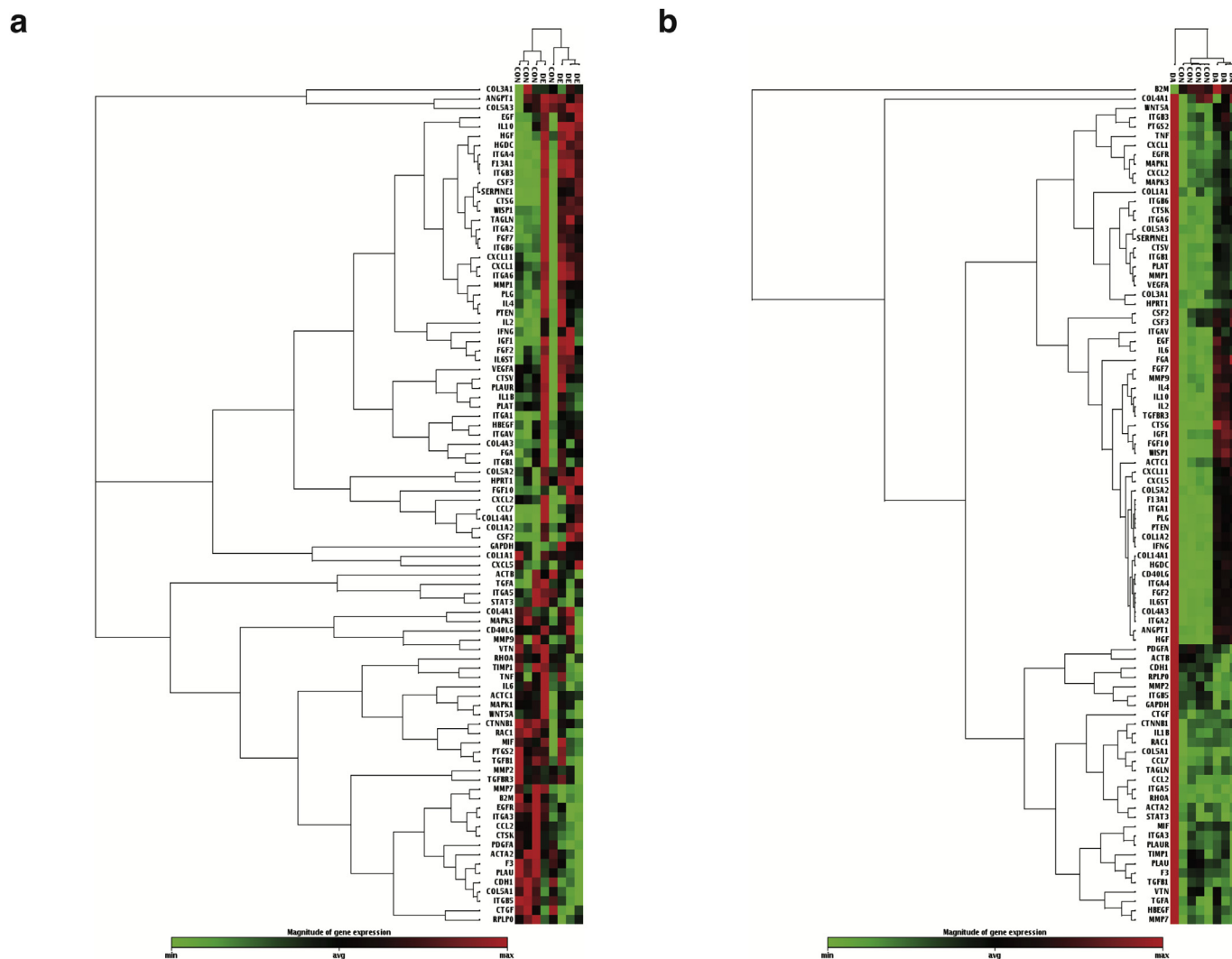


Fig. 5. Heat map of the expression of genes related to human wound healing in HaCaT cells treated with decursin and decursinol angelate. All signals were compared to a medium value, and the colors represent the magnitude of gene expression (scale shown at the bottom). a. Expression of genes related to human wound healing in HaCaT cells treated with decursin. b. Expression of genes related to human wound healing in HaCaT cells treated with decursinol angelate.

both regulate genes related to human wound healing but that their targets differ. Decursin upregulated expression of CTSG, F13A1, and ITGB3 by more than 10-fold, which encode extracellular matrix (ECM) remodeling enzymes and cell adhesion molecules. By contrast, decursinol angelate upregulated expression of COL1A2, CTSG, FGF10, HGF, IL10, and HGDC by more than 10-fold, which encode ECM structural proteins, inflammatory cytokines, and growth factors (Fig. 5).

4. Discussion

Many coumarins found in medicinal plants have diverse effects such as anti-inflammatory and anti-cancer activities as well as improvement of blood circulation, memory, and wound healing [1]. Decursin and decursinol angelate are the main coumarins in *A. gigas* Nakai and have a variety of biological effects [5].

This study focused on the wound healing effects of purified decursin and decursinol angelate, decursin complexes, and a nano-emulsion of these complexes. We also investigated which genes underlie these effects in HaCaT cells treated with purified decursin and decursinol angelate. Wound healing was improved by

treatment with decursin, decursinol angelate, and decursin complexes up to a threshold concentration, but was inhibited by treatment with higher concentrations. The MTT assay demonstrated that this inhibition of wound healing was not due to decreased proliferation of HaCaT cells (Supplementary Fig. 3). At higher concentrations, decursin, decursinol angelate, and decursin complexes may form oil droplets because they are relatively hydrophobic [11–13], thereby reducing their bioavailability to HaCaT cells. This would explain why treatment with higher concentration of these reagents did not improve wound healing. The nano-emulsion of decursin complexes enhanced wound healing at lower concentrations than decursin, decursinol angelate, and decursin complexes, indicating that its bioavailability was higher.

Phosphorylation of EGFR in human keratinocytes is a first step in skin wound healing, followed by modulation of mitogen-activated protein kinase, ERK1/2, and protein kinase B phosphorylation [14]. Phosphorylation of ERK1/2 is important for cell survival, proliferation, and inhibition of apoptosis. Therefore, we monitored EGFR and ERK1/2 phosphorylation via immunoblotting in HaCaT cells treated with decursin complexes. This revealed that treatment with decursin complexes induced phosphorylation of ERK1/2 but

not of EGFR. Moreover, this treatment could not make endosomes by activation of EGFR in EGFR biosensor, in contrast with hEGF treatment. These results indicate that the effect of decursin and decursinol angelate on skin wound healing is not due to EGFR phosphorylation but is controlled by ERK1/2 phosphorylation.

We next analyzed the transcription of human wound healing-related genes in HaCaT cells treated with decursin and decursinol angelate. A total of 41 of 85 genes connected with human wound healing were upregulated by treatment with decursin and decursinol angelate. Among these, six genes were upregulated more by decursin than by decursinol angelate, whereas 24 genes were upregulated more by decursinol angelate than by decursin. The genes that were upregulated more by decursin mainly encode ECM remodeling proteins and inflammatory cytokines, whereas those that were upregulated more by decursinol angelate mainly encode growth factors. This suggests that although decursin and decursinol angelate have very similar structures, their targets differ. Expression of genes encoding ECM remodeling proteins, inflammatory cytokines, and growth factors is a crucial step in human wound healing [9], and simultaneous expression of these genes can speed up this process. Thus, decursin and decursinol angelate can accelerate wound healing by simultaneously upregulating expression of such genes.

Competing financial interests

The authors declare no competing financial interests.

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Transparency document

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.bbrc.2018.04.031>.

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