

Evaluation of the effect of Thai breadfruit's heartwood extract on the biological functions of fibroblasts from wrinkles

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Synopsis

In previous studies, extract from *Artocarpus incisus*'s heartwood (breadfruit tree) had antioxidant and antimelanogenic activities. Here, we investigated the extract's action on facial skin fibroblasts from wrinkled skin and nonwrinkled skin biopsies, particularly in the production of type I procollagen and metalloproteinase-1 (MMP-1) and in the reorganization of collagen fibers. We found that the extract at a concentration of 50 µg/ml significantly enhanced percent viability and proliferation of wrinkled-skin fibroblasts. Flow cytometry showed that a 3.6-fold increased proportion of the wrinkled-skin fibroblasts were in their cell cycle S-phase, indicating increased proliferation. Type I procollagen synthesis by wrinkled-skin fibroblasts was augmented by the extract. Nonwrinkled-skin fibroblasts had higher synthesis and were unaffected by the extract. MMP-1 secretion was greater for wrinkled-skin fibroblasts, but the extract decreased its secretion for both fibroblasts samples. Fibroblasts were incorporated in collagen lattice disks. Lattices with nonwrinkled-skin fibroblasts contracted uniformly by 56% after a three-day culture and the extract had little effect. However, wrinkled-skin fibroblast lattices failed to show appreciable contractions (to 12% after three days). But remarkably, the extract conferred an ability of the wrinkled-skin fibroblast lattices to fully contract (to 53%). This shows that wrinkled-skin fibroblasts have the ability to reorganize collagen but that the extract can reactivate this latent potential. Our findings for the first time reveal that *A. incisus*'s heartwood extract reversed the fibroblast deficiencies in the metabolism and reorganization of collagen and may underlie a wrinkle treatment.

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INTRODUCTION

Aging is now generally regarded as a failure of an organism to repair tissue at the same rate as it is damaged (1,2). The changes occurring during aging are associated with alterations in skin appearance due to loss of tensile strength, with wrinkle formation, increased fragility, and decreased epidermal moisture content. Among these effects, wrinkles are an important indicator of aging and an interesting field in cosmetic dermatology. The appearance of wrinkles is related to an inflexibility of the skin coupled with a slackening of the dermis (3,4). This arises from a reduced production of collagen fibers accompanied by the degeneration of the surrounding collagenous network (5–7). Resident fibroblasts are intimately involved in the decreased synthesis of collagens such as type I procollagen and an increase in the degradation of collagens. This is through increased collagenases such as matrixmetalloproteinase-1 (MMP-1), which occurs as fibroblasts age (8–10). In addition, aged fibroblasts lose their capacity to adhere and move over collagen fibers, thereby limiting their ability to reorganize and reorient dermal tissue including collagen fibers (11–13). For this reason, the improvement of fibroblast activities in terms of dermal tissue biosynthesis and reorganization may be useful for a variety of cosmetic and therapeutic applications.

It has been demonstrated that agents such as *all-trans* retinol (14–17) and vitamin C (18,19) can stimulate collagen production and suppress MMP activity. In addition, it has been reported that vitamin C and soy peptides can restore the capacity of fibroblasts to realign the collagen fibers, which in turn improves locomotion, distribution, and adhesion of fibroblasts in the collagen matrix (11). These findings indicate that aged-related fibroblast dysfunction is, at least in part, reversible. Thus it would be realistic to develop new agents whose activities are similar to those of retinol- and/or soy proteins that could be applied as cosmetics.

In the cosmetics industry, there is demand for multifunctional and efficacious products based on real innovation and aligned with trends in the cosmetics market. For example, natural products with antioxidant, antityrosinase and antiaging activities have been sought for the treatment of photoaging, unwanted skin pigmentation, and wrinkles. Recently, we found that diethyl ether extracts using the heartwood from the Moraceae family, particularly *Artocarpus incisus* (breadfruit or Sa-kae in Thai) exhibited antioxidant activity in a dose-dependent manner using the DPPH assay (20). Furthermore, it has been reported that a crude extract as well as some purified compounds isolated from *A. incisus*'s heartwood inhibited melanin production (20–22). In the present study, therefore we compared the effects of the extract on some biological functions of fibroblasts from nonwrinkled and wrinkled skin that were obtained from a biopsy of skin at the outer corner of the eye. The fibroblast functions assessed included the stimulatory effect on proliferation, production of type I procollagen, and the inhibitory effect on the production of the major collagen-degrading enzyme (MMP-1). In addition, we studied the effect of the extract on the ability of “wrinkled-skin fibroblasts” to reorganize collagen fibers in a collagen lattice. The effect can be measured as a contraction of the collagen lattice, which thus indicates reorganization capacity (23,24). The results obtained from our study first revealed the biological effects *A. incisus*'s heartwood extract on human wrinkled-skin fibroblasts that were distinct from non-wrinkled skin cells. They also show that *A. incisus*'s heartwood extract could have potential cosmetic applications.

MATERIALS AND METHODS

PLANT MATERIAL AND THE EXTRACTION PROCESS

The heartwood of *A. incisus* was collected from Phitsanulok Province, Thailand. The heartwood portion of *A. incisus* was chipped and dried at 50°C by using a hot-air oven. Then the dried-chipped heartwood was milled into a powder. Five-hundred grams of the *A. incisus* powder was macerated with 800 ml of diethyl ether (LabScan Asia, Co. Ltd., Bangkok, Thailand) at room temperature for two days, as used previously with slight modification (20). The mixture was filtered through a cloth to remove particulates, and then the diethyl ether was removed by evaporation with a vacuum evaporator set at 33°C. The resultant powder was stored in a tight amber glass at -20°C for further studies.

QUALITY CONTROL OF THE EXTRACT

To control the extract quality of each batch, the content of the artocarpin, a major component of the *A. Incisus* heartwood extract, was determined by using isocratic high-performance liquid chromatography (HPLC). The artocarpin was provided by Assist Prof. Atawit Somsiri, Faculty of Pharmaceutical Sciences, Naresuan University (25). The HPLC instrument consisted of an SPD-10M10AVP diode array detector and an SCL-10A central unit (Shimadzu Co., Ltd., Kyoto, Japan). An Alltima 250 × 4.60-mm column containing 5 µm of C18 was the stationary phase (Alltech Associates Inc. Corporation, Illinois). The mobile phase was methanol (80 parts) (HPLC grade, LabScan Asia Co. Ltd.) and water (20 parts). The flow rate was 1 ml/min and the injection volume was 20 µl. The quantification of artocarpin was based on peak area at 282 nm. Determinations were performed in triplicate.

EFFECTS OF EXTRACT ON THE VIABILITY AND PROLIFERATION OF HUMAN FIBROBLASTS

Cells and treatment. Fibroblasts were obtained from a healthy female aged 58 years. Dermal tissue had been collected aseptically from the nonwrinkled and the wrinkled facial areas situated at the outer corner of the eye. Three-millimeter disks of skin were cut using a biopsy punch. Two to three skin disks were then placed in a 25-cm² flask and subsequently incubated for 1 h at 37°C with a humid atmosphere containing 5% CO₂. After incubation, the tissue disks could well attach on the wall of the culture flask. The culture medium consisted of DMEM (PanTM Biotech GmbH, Aidenbach, Germany), 10% FBS (PanTM Biotech GmbH), and 1% of a stock penicillin/streptomycin solution (PanTM Biotech GmbH); 3 ml was added to each flask. After incubation for three weeks at 37°C with 5% CO₂, the fibroblast cells had migrated from the original site. The fibroblast cells were then detached by trypsinization using trypsin-EDTA solution (PanTM Biotech GmbH) and seeded at 1 × 10⁴ cells/cm² in 75-cm² flasks using the same medium. Passage numbers 5 to 7 were used in this study.

For the cell treatment procedures, the cell suspension from nonwrinkled or wrinkled skin was transferred from the 75-cm² flask into a 96-well plate at a density of 5 × 10³ cells/well or a 12-well plate at a density of 4 × 10⁴ cells/well for the cell viability or cell proliferation

studies, respectively. Cells were initially cultured in the culture medium at 37°C for 24 h. Then the medium in each well was replaced with serum-free DMEM containing various concentrations of the extract (0.5, 1, 2, 5, 10, 20, and 50 µg/ml). Dimethyl sulfoxide (DMSO, 99.5% GC, Sigma-Aldrich, Inc., Missouri) was used to enhance the solubility of the extract in DMEM, and the amount used was not more than 0.1% in the final concentration. There were three groups of cultures: (i) serum-free DMEM (untreated group), (ii) serum-free DMEM + DMSO (0.1%, control group), and (iii) serum-free DMEM + extract (treated group).

Cell viability and proliferation assay. The activity of mitochondrial dehydrogenases was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide test (MTT, Sigma-Aldrich, Inc.). This test is an alternative method to measurement of the cellular viability as mitochondrial dehydrogenases from living cells are able to convert soluble MTT to an insoluble formazan via a reduction reaction. After incubation under 5% CO₂ at 37°C for 24, 48, or 72 h, the cell-free supernatants were removed and replaced with 100 µl of serum-free DMEM. Fifteen microliters of MTT solution (5 mg/ml in PBS) was added to each well. After incubation for 4 h, 100 µl of extraction buffer consisting of 10% w/v sodium dodecyl sulfate (Sigma-Aldrich, Inc.) in 0.5 M N,N-dimethylformamide (Sigma-Aldrich, Inc.) was added. The samples were then incubated overnight under 5% CO₂ at 37°C. The optical density of the converted dye was measured at 510 nm by using a Labsystems Multiskan RC 96-well microplate reader (Thermo LabSystems, Inc., Massachusetts). Optical density was adjusted to 100% using the untreated cell groups, and the cell viability results were thus expressed as a percentage. The measurements were performed in triplicate.

In this study, cell proliferation was determined by counting the number of viable cells in the individual wells by using the trypan blue exclusion test. Cells were treated with the extract or DMSO for 1, 3, 6, or 10 days. The medium was replaced every three days with fresh medium of the appropriate type. The study was performed in triplicate to obtain the average number of viable cells. The appearance of the cells was verified by microscopic examination.

Cell cycle analysis. After 72 hr, the proportion of cells in each of the cell cycles, (G1, S, and G2) was determined by staining the cell DNA with propidium iodide (PI). Briefly, the cells were detached by trypsinization and washed twice with phosphate buffer saline (PBS without Ca²⁺ and Mg²⁺, PanTM Biotech GmbH) containing 2 mM of disodium ethylenediamine tetracetic acid (Na EDTA). The cells were fixed overnight with cold absolute ethanol and then stained with a solution containing 5 µl of PI (0.1 mg/ml, Sigma-Aldrich, Inc.), 1 µl of RNase (1 mg/ml, Sigma-Aldrich, Inc.), and 49 µl of 2 mM Na EDTA in PBS. After 5-min incubation at room temperature in the dark, fluorescent cells were sorted in a CytomicsTM FC 500 flow cytometry system equipped with a 488-nm argon laser (Beckman Coulter, Inc., California). The data were analyzed on the RXP software.

EFFECTS OF THE EXTRACT ON PRODUCTION OF TYPE I PROCOLLAGEN AND MATRIX METALLOPROTEINASE-1 (MMP-1) BY HUMAN FIBROBLASTS

Cells and treatment. Fibroblasts from cultures of the same explants as above (passage numbers of 5 to 7) were used in these experiments. Before being treated, the cell

suspension was transferred from the 75-cm² flask into 12-well plate at a density of 1×10^5 cells/well of DMEM with FBS and antibiotics. The cells were incubated at 37°C for 24 h in a humid atmosphere containing 5% CO₂. There were two groups of cultures: (i) serum-free DMEM + DMSO (0.1%, control group) and (ii) serum-free DMEM + extract (treated group). The cells continued to be incubated at 37°C with 5% CO₂ for 72 h.

Type I procollagen and MMP-1 assay. In this study, the procollagen assay used an antibody against the C-terminal propeptide region that is part of the transcribed collagen protein that is then proteolytically cleaved after secretion. Thus, this assay is a measure of newly synthesized collagen. After incubation with the extract for 72 h, the cell-free supernatant was collected, stored at -80°C, and then assayed later. The amount of type I procollagen was measured by using a commercial human procollagen type-I C-peptide EIA kit (Takara Bio Inc., Shiga, Japan). The same samples were also assayed for MMP-1 (interstitial collagenase) by using a commercial human MMP-1 ELISA kit (RayBiotech, Inc., Georgia, USA). The levels of type I procollagen and MMP-1 were normalized against a standard dose-response curve based on the absorption at the wavelength of 450 nm using a Labsystems Multiskan RC 96-well microplate reader. The determinations were performed in triplicate.

EFFECT OF THE EXTRACT ON CONTRACTION OF A FIBROBLAST-EMBEDDED COLLAGEN LATTICE

Fibroblast-embedded collagen lattice preparation. A three-dimensional collagen lattice was prepared according to the previous studies (11,26) with modification. Fibroblasts from nonwrinkled and wrinkled skin were collected from the same explants as mentioned above. Cells at passage 7 were used to prepare the fibroblast-embedded lattice. Briefly, the disk-shaped matrix consisted of 1.98 ml of concentrated DMEM 1.96 X (GIBCO™, Invitrogen™ Life Technologies, California) containing 50 µg/ml of the extract (treated group) or 0.1% of DMSO (control group), 1.5 ml of rat tail type I collagen (Institut de Biotechnologies Jacques Boy, Reims, France), 0.25 ml of 0.1 N NaOH (Prolabo, Fontenay-Sous-Bois, France), 0.17 ml of 7.5% NaHCO₃ (Pan™ Biotech GmbH), 0.50 ml of FBS (Pan™ Biotech GmbH), and 0.5 ml of cell suspension (8×10^5 cells/ml). Matrices were prepared in 60-mm Petri dishes (Falcon bacteriological dishes, Elvetec Services, Clemont-Ferrand, France) and then placed in a 37°C incubator in a humid atmosphere containing 5% CO₂. Matrix disks were prepared in triplicate for each group and experiments were performed in duplicate.

Contraction capacity determination. The contraction capacity of the fibroblast-embedded lattice was visually determined from the lattice diameter. To measure matrix diameters, they were placed on a transparent metric ruler on a dark background. The matrix diameters were measured over the seven-day culture period.

STATISTICAL ANALYSIS

All quantitative data reported here are expressed as means of the samples for each treatment. Student's unpaired *t*-test was used for comparison between the two groups. *P* < 0.05 was considered significant.

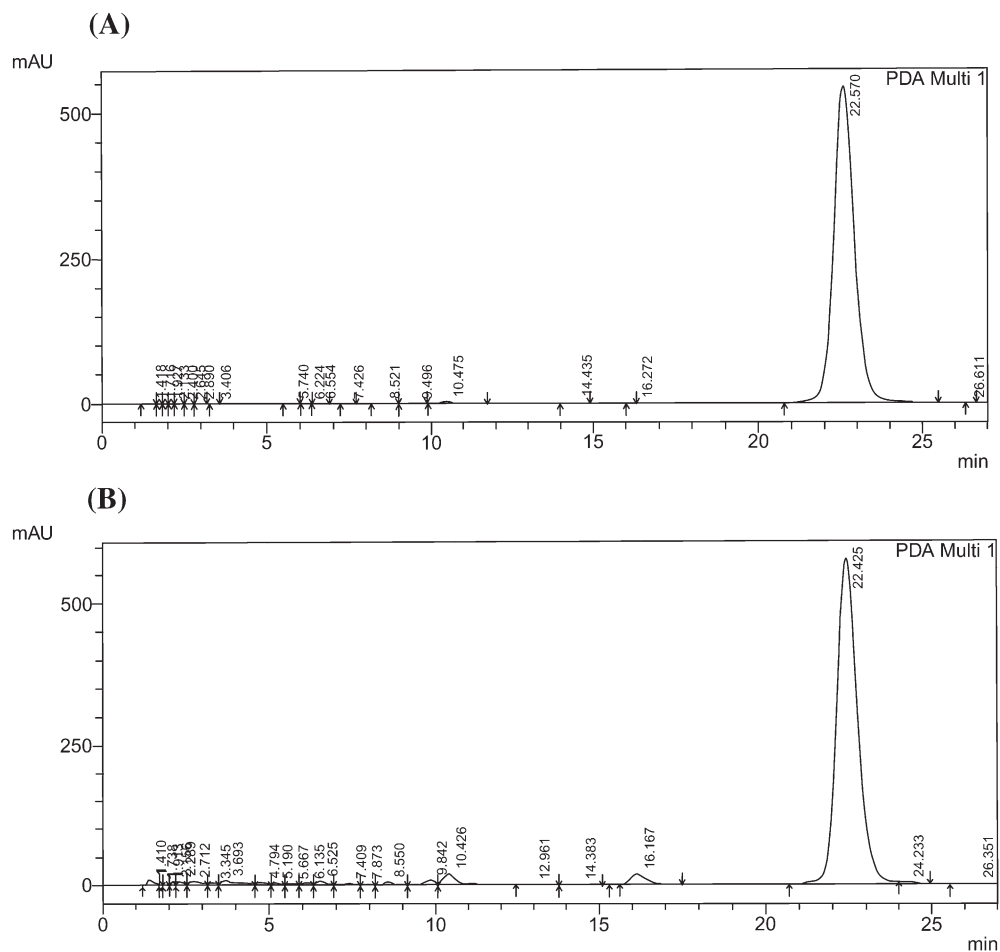
RESULTS AND DISCUSSION

THE APPEARANCE AND ARTOCARPIN CONTENT OF THE EXTRACT

Extraction of diethyl ether provided a yellow solid powder. The HPLC chromatograms of the artocarpin standard and the artocarpin contained in the extract are shown in Figure 1A and 1B, respectively. The amount of artocarpin contained in the extract was $44.5 \pm 0.1\%$ (w/w). This finding coincided with our previous study indicating 45.2% (w/w) of artocarpin in the ether extract (20).

EFFECTS OF THE EXTRACT ON THE VIABILITY AND PROLIFERATION OF HUMAN FIBROBLASTS

To investigate the effect of the extract on cell viability, the fibroblasts were treated with various concentrations (0.5–50 $\mu\text{g/ml}$) of the extract for 24, 48, or 72 h. As shown in Figure 2A, the extract did not affect the viability of nonwrinkled-skin fibroblasts.



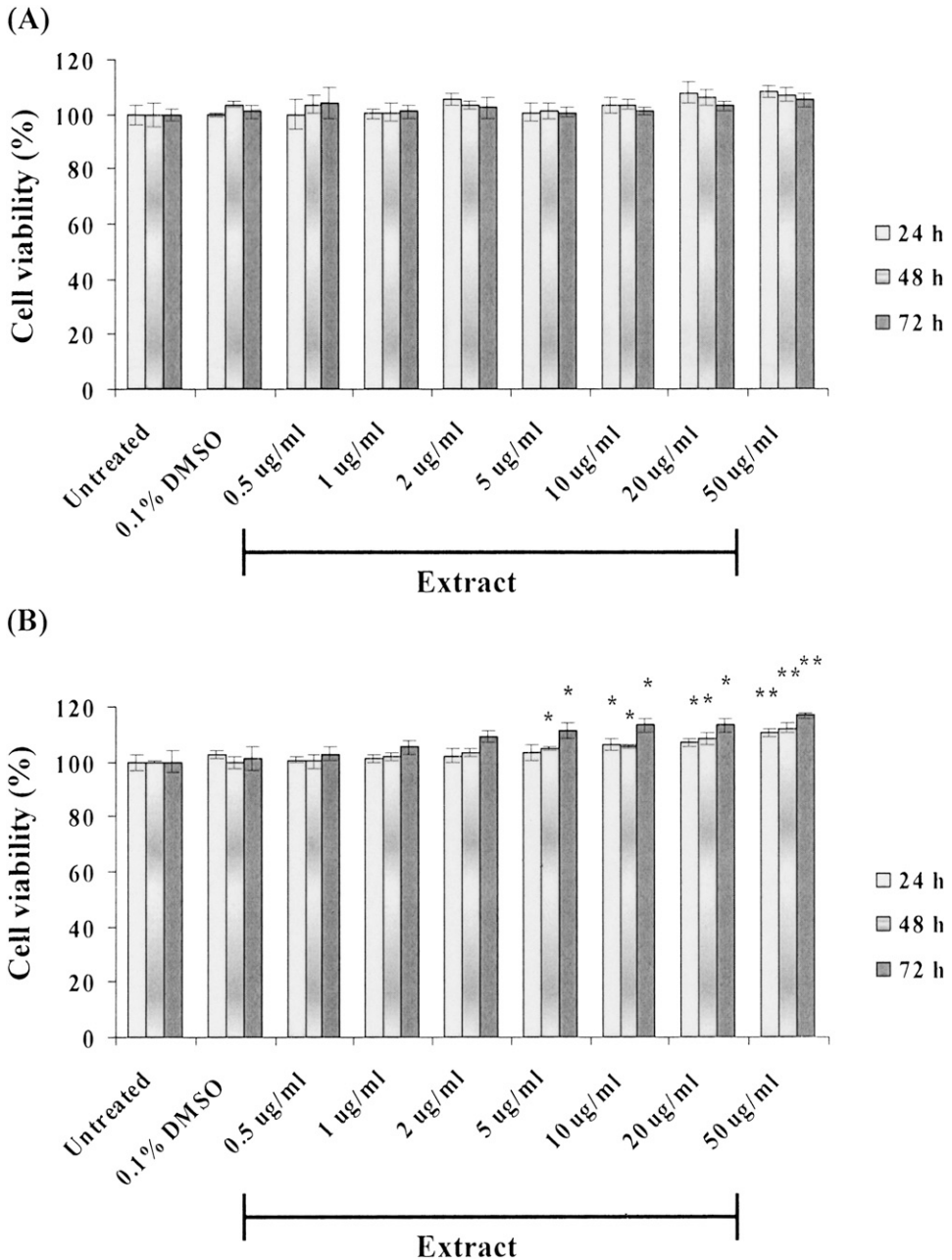


Figure 2. Effects of *A. incisus* extract on viability of fibroblasts (A) from nonwrinkled skin and (B) from wrinkled skin. Fibroblasts were treated with 0.1% DMSO or the extract at concentrations in the range of 0.5–50 $\mu\text{g}/\text{ml}$ for 24, 48, and 72 h. Results are expressed as percentage of cell viability as compared to untreated cells for which the optical density was adjusted to 100%. Each bar represents mean \pm S.D. of triplicate study; * $p < 0.05$ and ** $p < 0.01$ denote significant differences when compared to untreated cells (Student's *t*-test).

However, a significant increase in percent viability as compared with the untreated cells was found in the wrinkled-skin fibroblasts treated with the extract at concentrations of 10 $\mu\text{g}/\text{ml}$ ($p < 0.05$), 20 $\mu\text{g}/\text{ml}$ ($p < 0.05$), and 50 $\mu\text{g}/\text{ml}$ ($p < 0.01$) for 24 h (Figure 2B). An increase in the incubation time to 48 or 72 h resulted in significant increases in cell viability after treatment with the extract at a lower concentration (5 $\mu\text{g}/\text{ml}$, $p < 0.05$). This effect might be due to increased fibroblasts numbers, and so this was tested by counting cell numbers. Cells were treated with the extract at concentrations of 20 $\mu\text{g}/\text{ml}$ or 50 $\mu\text{g}/\text{ml}$ for 1, 3, 6, or 10 days and directly counted by using the trypan blue exclusion method. Focusing on untreated cells, the proliferation of cells from wrinkled skin was lower than that of cells from nonwrinkled skin, particularly during the first three days of the study. Treatment with 50 $\mu\text{g}/\text{ml}$ of extract resulted in a higher proliferation of the cells from wrinkled skin during the incubation period as compared with the untreated cells ($p < 0.05$), as shown in Figure 3B. Such a phenomenon was not observed in the fibroblasts from nonwrinkled skin (Figure 3A). The morphology of the fibroblasts did not change compared to that of the untreated cells (Figure 4A–D). The fibroblasts still retained the typical spindle-shape after treatment with the extract at the highest concentration used in this study (50 $\mu\text{g}/\text{ml}$).

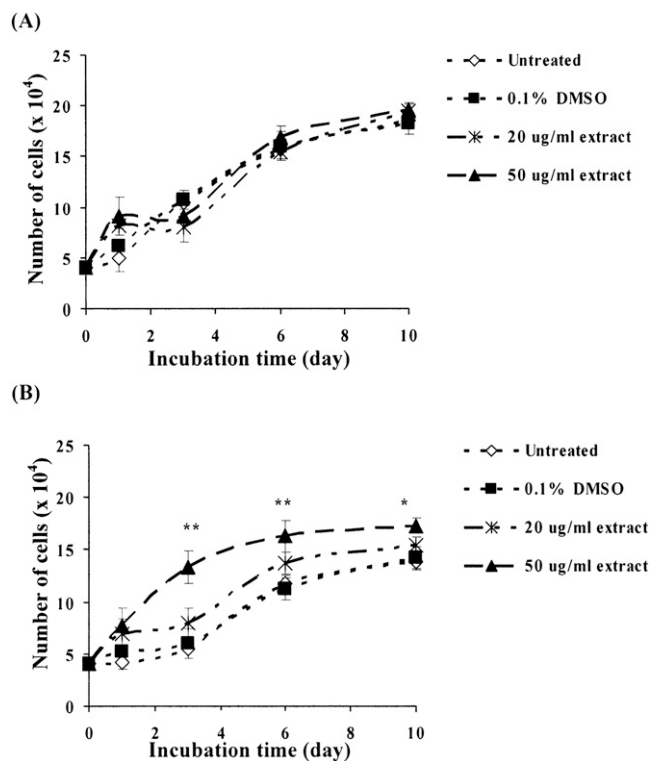


Figure 3. Effects of *A. incisus* extract on proliferation of fibroblasts (A) from nonwrinkled skin and (B) from wrinkled skin. Fibroblasts were treated with 0.1% DMSO or the extract at concentrations of 20 $\mu\text{g}/\text{ml}$ or 50 $\mu\text{g}/\text{ml}$ for 1, 3, 6, or 10 days. Results are expressed as the number of cells. Each point represents mean \pm S.D. of triplicate study; * $p < 0.05$ and ** $p < 0.01$ denote significant differences when compared to untreated cells (Student's *t*-test).

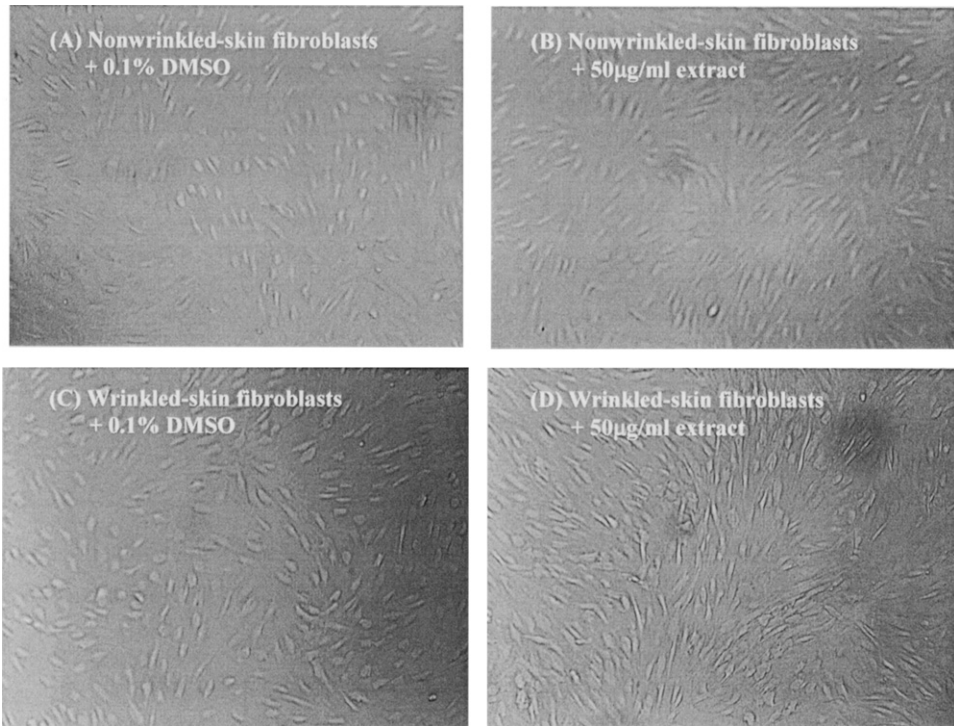


Figure 4. Morphology of nonwrinkled-skin fibroblasts (A) treated with 0.1% DMSO or (B) treated with 50 µg/ml of extract; and wrinkled-skin fibroblasts (C) treated with 0.1% DMSO or (D) treated with 50 µg/ml of extract for 72 h (at magnification of 10×).

Table I

Percentage of Fibroblasts from Nonwrinkled and from Wrinkled Skin at Different Stages of the Cell Cycle (G1, S, and G2) after Treatment with 50 µg/ml of *A. incisus* Extract for Three Days

Cell type	Treatment	G1 (%)	S (%)	G2 (%)
Nonwrinkled-skin fibroblasts	0.1% DMSO	86.59	3.53	9.88
	50 µg/ml extract	76.80	5.99	17.21
Wrinkled-skin fibroblasts	0.1% DMSO	89.77	4.66	5.57
	50 µg/ml extract	67.94	17.82	14.24

In addition, a cell cycle study of the fibroblasts treated with the extract was conducted by flow cytometry. The cell cycle stages in the control (with 0.1% DMSO) and the treated condition (with 50 µg/ml extract) are summarized in Table I, and histograms of the flow cytometric data are shown in Figure 5A–D. In comparison to the fibroblasts from wrinkled skin, the number of cells in the G2 phase of the fibroblasts from nonwrinkled skin was about twofold higher. The extract increased the proportion of wrinkled-skin fibroblasts treated to be in the G2 phase by about 2.6-fold. The same trend was found for extract-treated nonwrinkled-skin fibroblasts, but the effect was smaller (1.7-fold). A concomitant increase in cell numbers in the S phase by about 3.6-fold was seen for wrinkled-skin fibroblasts treated with the extract. This reciprocal relationship between the S and G2 phases indicates that the extract could have increased proliferation of wrinkled-skin

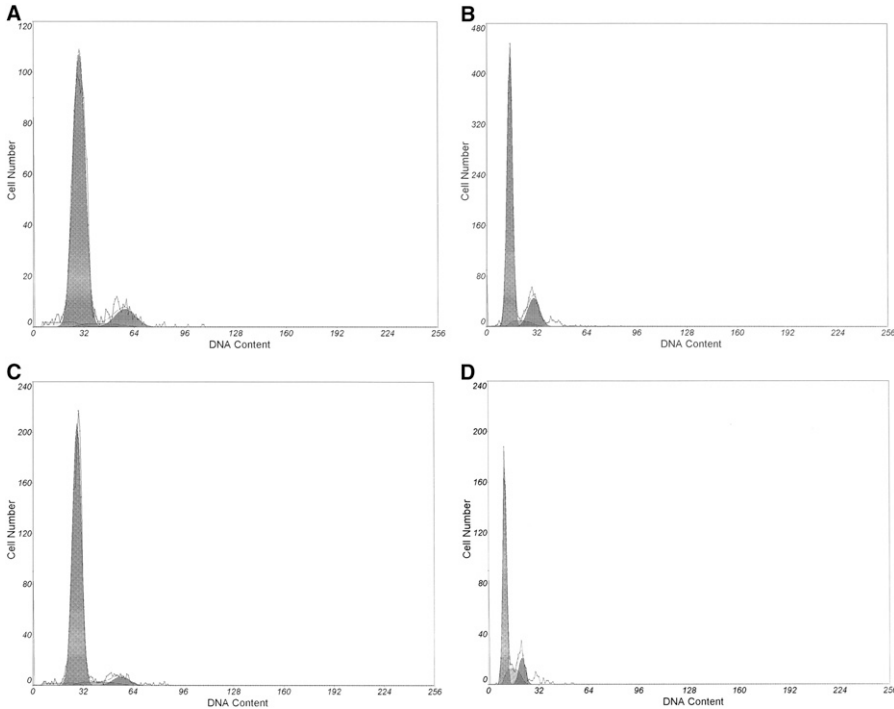


Figure 5. Cell-cycle analysis of nonwrinkled-skin fibroblasts (A) treated with 0.1% DMSO or (B) treated with 50 µg/ml of extract; and wrinkled-skin fibroblasts (C) treated with 0.1% DMSO or (D) treated with 50 µg/ml of extract for 72 h.

fibroblasts by driving the cells from the G1 phase into the S phase. Taken together, this suggests that the extract enhances cell proliferation and promotes viability during the division of fibroblasts from wrinkled skin.

EFFECTS OF THE EXTRACT ON PRODUCTION OF TYPE I PROCOLLAGEN AND MATRIX METALLOPROTEINASE-1 (MMP-1) BY HUMAN FIBROBLASTS

Wrinkled-skin fibroblasts produced less type I procollagen than nonwrinkled-skin fibroblasts (76% greater, $p < 0.01$) as shown in Figure 6A. In contrast, wrinkled-skin fibroblasts accumulated more MMP-1 in the culture medium ($p < 0.05$, Figure 6B). Nevertheless, the extract suppressed MMP-1 synthesis by fibroblasts from both skin types, but the effect on procollagen was confined to wrinkled-skin cells.

It is widely known that skin aging correlates with the loss of dermal connective tissue, subsequently resulting in the development of skin wrinkles. It has been reported that the loss of type I collagen in aged skin is due to a progressive increase in MMP synthesis and hence increased degradation (9,19). This is further aggravated by declining collagen synthesis (14,27). MMP-1 one of several MMP cutaneous collagenases, but it is expressed much less in the young (28–30) than in the aged, where it becomes the more dominant degrading enzyme (31). This coincides with the present study showing less MMP-1 synthesis in nonwrinkled-skin fibroblasts. Additionally, the extract at

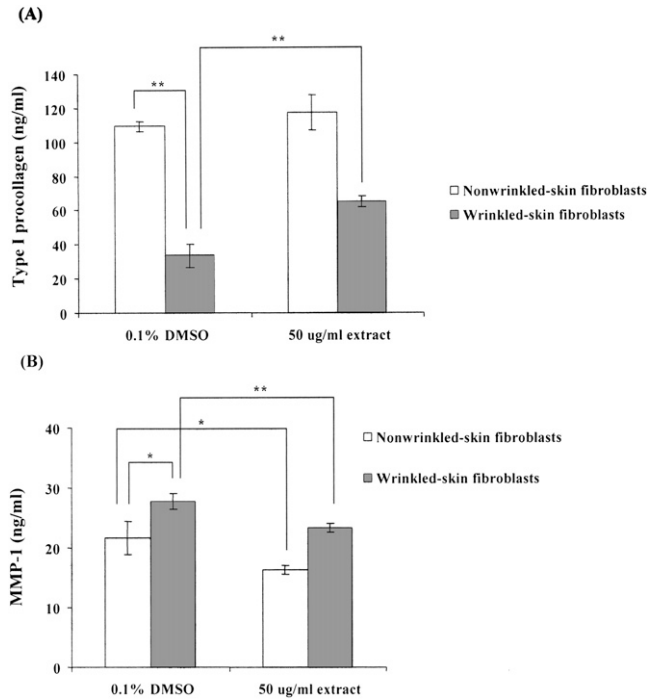


Figure 6. Effects of *A. incisus* extract on (A) type I procollagen or (B) MMP-1 production by nonwrinkled-skin and wrinkled-skin fibroblasts. Fibroblasts were treated with 0.1% DMSO (control) or 50 µg/ml of extract for 72 h. Each bar represents mean \pm S.D. of triplicate study. * $p < 0.05$ and ** $p < 0.01$, when compared with control values (Student's t -test).

the concentration used here (50 µg/ml) was found to inhibit elaboration of this key enzyme, MMP-1. Concomitantly, the same extract concentration markedly stimulated type I procollagen synthesis by wrinkled-skin fibroblasts. Such a stimulation of procollagen synthesis might be caused, at least partially, by an increasing cell proliferation, since enhancement of wrinkle-skin fibroblast proliferation by the extract was found. From these combined effects, the extract could improve collagen metabolism by dermal fibroblasts.

EFFECT OF THE EXTRACT ON CONTRACTION OF THE FIBROBLAST-EMBEDDED COLLAGEN LATTICE

In addition to improved collagen metabolism, the reorganization and reorientation of collagen fibers are necessary to minimize the development of skin wrinkles (12,32). Such remodeling of collagen fibers is governed by fibroblasts and, in turn, is shown to affect the locomotion and attachment of fibroblasts (11,33). Figure 7A shows the extent of contraction (initial diameter decreased by 56%) of lattices populated with nonwrinkled-skin fibroblasts vigorously for the first three days; after that, the contraction progressed more slowly. In comparison, the lattices containing the wrinkled-skin fibroblasts produced a slight contraction (the initial diameter decreased by only 12% after three days), but most importantly, the extract restored the capacity of the lattice with the wrinkled-skin fibroblasts to contract in a manner similar to that of the lattices containing nonwrinkled-skin

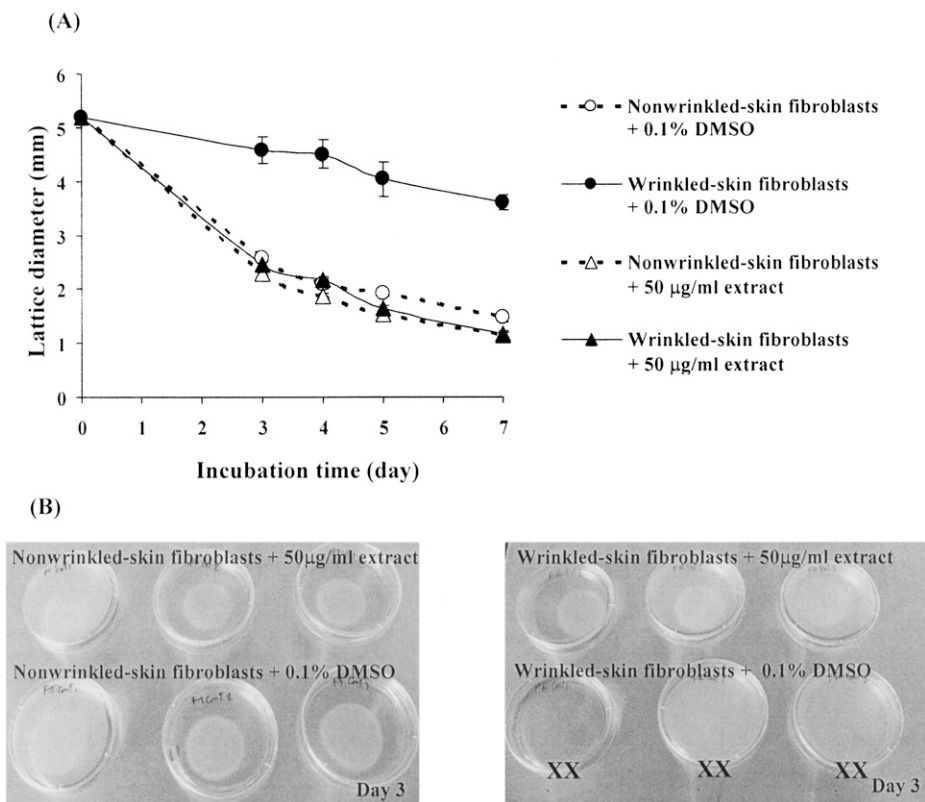


Figure 7. Effect of *A. incisus* extract on the contractile capacity of nonwrinkled-skin and wrinkled-skin fibroblasts. Fibroblasts embedded in the collagen lattice were treated with 0.1% DMSO (control) or 50 µg/ml extract. (A) The diameter of each lattice was measured over seven days of culture. Each point represents mean \pm S.D. of three samples in duplicate. (B) Photographs of collagen lattices floating singly in the culture dishes at day 3. In dishes marked 'XX', the boundary of the lattice is difficult to discern because it fills of the dish.

fibroblasts (by 53% at three days). Furthermore, the contraction was uniform as shown by the disk shape of the lattices (Figure 7B). These observations show that our extract improved the contractile ability and reorganization of the collagen fibers shown by the wrinkled-skin fibroblasts. Taken together with the above-mentioned results, these observations suggest that the extract affects not only enhancement of dermal collagen production, but also the restoration of dermal collagen produced by fibroblasts. However, it is important to note that a limitation of this study is the source of the fibroblasts used. As the study was performed with fibroblasts obtained from only one individual, further investigation in fibroblasts from various sources is necessary to ensure the efficacy of the extract on the restoration of fibroblast functions.

CONCLUSIONS

Nowadays, the botanical extract is playing an increasingly important role in cosmetics. The isolation and purification of active ingredients is sometimes unnecessary for cosmetics, and purification may lead to a loss of biological activity and may cause toxicity. Our

previous study reported the effects of the crude extract of *A. incisus*'s heartwood on tyrosinase, melanogenesis, and oxidation activities. Therefore, this study focused on the ability of the extract to improve the functions of fibroblasts from wrinkled-skin biopsies. These functions include cell proliferation, type I procollagen and MMP-1 production, and the capacity to reorganize collagen fibers. We found that the aged-related decrease in the functional activities of fibroblasts from wrinkles could be reversible by treatment with the extract from *A. incisus*'s heartwood. However, these findings on cell-culture fibroblasts do not prove that the extract will decrease wrinkles in humans. Clinical studies in human subjects will be needed to determine the efficacy of the extract on wrinkle reduction.

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REFERENCES

- (1) P. U. Giacomoni and G. Rein, A mechanistic model for the aging of human skin, *Micron*, **35**, 179–184 (2004).
- (2) V. N. Novoseltsev, J. Novoseltseva, and A. Yashin, A homeostatic model of oxidative damage explains paradoxes observed in the earlier aging experiments: A fusion and extension of older theories of aging, *Biogerontology*, **2**, 127–138 (2001).
- (3) D. Batisse, R. Bazin, T. Baldeweck, B. Querleux, and J.-L. Lévêque, Influence of age on the wrinkling capacities of the skin, *Skin Res. Technol.*, **8**, 148–154 (2002).
- (4) S. Diridollou, V. Vabre, M. Berson, L. Vaillant, D. Black, J. M. Lagarde, J. M. Grégoire, Y. Gall, and F. Patat, Skin aging: Changes of physical properties of human skin in vivo, *Int. J. Cosmet. Sci.*, **23**, 353–362 (2001).
- (5) J. H. Chung, J. Y. Seo, H. R. Choi, M. K. Lee, C. S. Youn, G. Rhie, K. H. Cho, K. H. Kim, K. C. Park, and H. C. Eun, Modulation of skin collagen metabolism in aged and photoaged human skin in vivo, *J. Invest. Dermatol.*, **117**, 1218–1224 (2001).
- (6) J. Varani, D. Spearman, P. Perone, S. E. G. Fligiel, S. C. Datta, Z. Q. Wang, Y. Shao, S. Kang, G. J. Fisher, and J. J. Voorhees, Inhibition of type I procollagen synthesis by damaged collagen in photoaged skin and by collagenase-degraded collagen in vitro, *Am. J. Pathol.*, **158**, 931–942 (2001).
- (7) E. F. Bernstein and J. Uitto, The effect of photodamage on dermal extracellular matrix, *Clin. Dermatol.*, **14**, 143–151 (1996).
- (8) G. Jenkins, Molecular mechanisms of skin aging, *Mech. Aging Dev.*, **123**, 801–810 (2002).
- (9) J. Varani, R. L. Warner, M. Gharaee-Kermani, S. H. Phan, S. Kang, J. H. Chung, Z. Q. Wang, S. C. Datta, G. J. Fisher, and J. J. Voorhees, Vitamin A antagonizes decreased cell growth and elevated collagen-degrading matrix metalloproteinases and stimulates collagen accumulation in naturally aged human skin, *J. Invest. Dermatol.*, **114**, 480–486 (2000).
- (10) M. D. West, The cellular and molecular biology of skin aging. *Arch. Dermatol.*, **130**, 87–95 (1994).
- (11) M. Jouandeaud, C. Viennet, S. Bordes, B. Closs, and P. Humbert, Comparison of the biomechanical and biosynthetic behavior of normal human fibroblasts and fibroblasts from a forehead wrinkle, *IFSCC Magazine*, **7**, 109–113 (2004).
- (12) E. Tamariz and F. Grinnell, Modulation of fibroblast morphology and adhesion during collagen matrix remodeling, *Mol. Biol. Cell*, **13**, 3915–3929 (2002).
- (13) H. U. Püschel, J. Chang, P. K. Müller, and J. Brickmann, Attachment of intrinsically and extrinsically aged fibroblasts on collagen and fibronectin, *J. Photochem. Photobiol. B*, **27**, 39–46 (1995).
- (14) H. S. Talwar, C. E. M. Griffiths, G. J. Fisher, T. A. Hamilton, and J. J. Voorhees, Reduced type I and type II procollagens in photodamaged adult human skin, *J. Invest. Dermatol.*, **105**, 285–290 (1995).

- (15) A. M. Kligman, D. Dogadkina, and R. M. Lavker, Effects of topical tretinoin on non-sun-exposed protected skin of the elderly, *J. Am. Acad. Dermatol.*, **29**, 25–33 (1993).
- (16) J. S. Weiss, C. N. Ellis, J. T. Headington, T. Tincoff, T. A. Hamilton, and J. J. Voorhees, Topical tretinoin improves photoaged skin: A double blind, vehicle-controlled study, *JAMA*, **259**, 527–532 (1988).
- (17) A. M. Kligman, G. L. Grove, R. Hirose, and J. J. Leyden, Topical tretinoin for photoaged skin, *J. Am. Acad. Dermatol.*, **15**, 836–859 (1986).
- (18) B. V. Nussgens, P. Humbert, A. Rougier, A. C. Colige, M. Haftek, C. A. Lambert, A. Richard, P. Creidi, and C. M. Lapiere, Topically applied vitamin C enhances the mRNA level of collagen I and III, their processing enzymes and tissue inhibitor of matrix metalloproteinase 1 in the human dermis, *J. Invest. Dermatol.*, **116**, 853–859 (2001).
- (19) G. J. Fisher, Z.-Q. Wang, S. C. Datta, J. Varani, S. Kang, and J. J. Voorhees, Pathophysiology of premature skin aging induced by ultraviolet light, *N. Eng. J. Med.*, **337**, 1419–1428 (1997).
- (20) P. Donsing, N. Limpeanchob, and J. Viyoch, Evaluation of the effect of Thai breadfruit's heartwood extract on melanogenesis-inhibitory and antioxidation activities, *J. Cosmet. Sci.*, **59**, 41–58 (2008).
- (21) K. Shimizu, R. Kondo, and K. Sakai, The skin-lightening effects of artocarpin on UVB-induced pigmentation, *Planta Med.*, **68**, 79–81 (2002).
- (22) K. Shimizu, R. Kondo, K. Sakai, S.-H. Lee, and H. Sato, The inhibitory components from *Artocarpus incisus* on melanin biosynthesis, *Planta Med.*, **64**, 408–412 (1998).
- (23) E. Bell, S. Stephanie, B. Hull, C. Merrill, S. Rosen, A. Chamson, D. Asselineau, L. Dubertret, B. Coulomb, C. Lapiere, B. Nussgens, and Y. Neveux, The reconstitution of living skin, *J. Invest. Dermatol.*, **81**, 2s–10s (1983).
- (24) E. Bell, B. Ivarssen, and C. Merrill, Production of a tissue-like structure by contraction of collagen lattices by human fibroblasts of different proliferative potential in vitro, *Proc. Natl. Acad. Sci. USA*, **76**, 1274–1278 (1979).
- (25) T. Pitaksuteepong, A. Somsiri, and N. Waranuch, Targeted transfollicular delivery of artocarpin extract from *Artocarpus incisus* by means of microparticles, *Eur. J. Pharm. Biopharm.*, **67**, 639–645 (2007).
- (26) C. Vinnat, V. Armbruster, A. C. Gabiot, T. Gharbi, J. Bride, and P. Humbert, Comparing the contractile properties of human fibroblasts in leg ulcers with normal fibroblasts, *J. Wound Care*, **13**, 358–361 (2004).
- (27) R. M. Lavker, Structural alterations in exposed and unexposed skin, *J. Invest. Dermatol.*, **73**, 59–66 (1979).
- (28) H. Watanabe, T. Shimizu, J. Nishihira, R. Abe, T. Nakayama, M. Taniguchi, H. Sabe, and T. Ishibashi, Ultraviolet A-induced production of matrix metalloproteinase-1 is mediated by macrophage migration inhibitory factor (MIF) in human dermal fibroblasts, *J. Biol. Chem.*, **279**, 1676–1683 (2004).
- (29) M. Brennan, H. Bhatti, K. C. Nerusu, N. Bhagavathula, S. Kang, G. J. Fisher, J. J. Voorhees, and J. Varani, Matrix metalloproteinase-1 is the major collagenolytic enzyme responsible for collagen damage in UV-irradiated human skin, *Photochem. Photobiol.*, **78**, 43–48 (2003).
- (30) J. Campisi, Replicative senescence: An old wives tale, *Cell*, **84**, 497–500 (1996).
- (31) A. J. Millis, M. Hoyle, H. M. McCue, and H. Martini, Differential expression of metalloproteinase and tissue inhibitor of metalloproteinase genes in aged human fibroblasts, *Exp. Cell Res.*, **211**, 90–98 (1992).
- (32) L. Rakic, C. M. Lapiere, and B. V. Nussgens, Comparative caustic and biological activity of trichloroacetic acid and glycolic acid on keratinocytes and fibroblasts in vitro, *Skin Pharmacol. Appl. Skin Physiol.*, **13**, 52–59 (2000).
- (33) H. K. Kleinman, M. L. McGarvey, and G. R. Martin, Role of endogenous collagen synthesis in the adhesion of human skin fibroblasts, *Cell Biol. Int. Rep.*, **6**, 591–599 (1982).