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Topical application of neem leaves prevents wrinkles formation in UVB-exposed hairless mice

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ABSTRACT

Azadirachta indica A. Juss. (Neem) has been used in India for the treatment of skin problems for centuries. However, no skin photoaging-related research has been performed with this agent. In the present study, neem leaf extract at 1, 10, and 50 μg/mL was investigated for its skin anti-aging effects in UVB-irradiated NHDFs and hairless mice. Regulation of molecular signaling pathways by neem leaf extract during UVB exposure was investigated in both in vitro and in vivo models. UVB-irradiated skin model treated with neem leaf extract showed increased type I procollagen and elastin as a result of enhanced synthesis via two pathways. Firstly, transforming growth factor TGF-β1 is up-regulated. Secondly, intracellular reactive oxygen species (ROS), transcription activator AP-1 protein expression, and MAPK are down-regulated. The down-regulation of signaling molecules caused the suppression of type I procollagen degrading enzymes such as matrix metalloproteinase-1 and type I procollagen synthesis inhibitor such as interleukin-6. In particular, topical application of neem leaf to UVB-irradiated hairless mice was shown to be very effective in treating the symptoms of skin aging such as wrinkles, thickening, water loss, and erythema. Therefore, our results indicated that neem leaf ethanolic extract is a promising anti-aging candidate for topical therapy products.

1. Introduction

Azadirachta indica A. Juss. (Neem or Margousier) famously used as a beauty aid in India, belongs to the mahogany family *Meliaceae* [1]. Indian people have used neem safely and effectively for more than 5000 years, and they refer to neem as "The Village Pharmacy" [2]. Additionally, it is used in cooking and in salads and is eaten as a fresh vegetable in Indian. The medicinal effects of neem were written about in the Vedas, the oldest book on earth, as "one that cures all ailments and ills", especially many common skin disorders [3]. Lotions, face creams, and nail polish are examples of external cosmetic products that contain neem extract while capsules and teas made from neem are effective for internal use [4].

Although neem has historically been demonstrated numerous uses, the research on the active compounds of neem has only been performed recently. More than 140 active ingredients have been extracted from the flowers, leaves, fruit, granular body, twigs, and roots of the neem plant; and their chemistry and structure have also been published [5–8]. In recent papers, the phytochemical components of neem were reported to include carotenoids, phenolic acids, and flavonoids that might provide high antioxidant activity, leading to potential activities in skin anti-aging [9–11].

Many studies on anti-aging mechanisms have been carried out using modern molecular markers. Aging skin shows erythema, swelling, wrinkle formation, sagging, skin thickening, and transepidermal water loss (TEWL) symptoms after UV-exposure due to the degradation of collagen and elastin that normally keep skin firm [12–14]. Fibroblasts present in the epidermis, have recently been shown to be involved in UVB-induced photoaging [15,16]. Collagen I is the most important protein shown to be involved in the regulation in aged-skin cells [17]. The synthesis of collagen I is known to require precursor procollagen type I synthesis by TGF-β/Smad signaling in fibroblasts [18]. On the other hand, collagen I can be degraded by UVB-induced ROS activation through two up-regulated pathways using the degrading enzymes of collagen I (MMPs) and a collagen synthesis inhibitor (IL-6), thereby leading to the activation of activator protein 1 (AP-1) [15,16].

However, there are no scientific data on the anti-aging activities of neem leaves. On the basis of the presence of neem's bioactive flavonoids

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and its traditional uses, the protective effects of neem leaves in skin anti-aging were demonstrated in UVB-irradiated normal human dermal fibroblasts (NHDFs) in vitro and via topical application on UVB-exposed hairless mice in vivo. Using a series of markers above, the NHDF cell signaling pathways and skin symptoms in the UVB-induced photoaging processes were investigated in this study.

2. Materials and Methods

2.1. Chemicals

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and penicillin/streptomycin were purchased from Gibco BRL (Grand Island, NY, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Neem leaves were purchased from Mountain Rose Herbs Company (Eugene, OR, USA). Rutin and PTU standard (purity 98%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Solvents were purchased from Samchun Chemicals (Samchun Pure Chemical, Korea). Enzyme-linked immunosorbent assay (ELISA) kits for MMP-1, IL-6, and TGF-β1 were purchased from R&D Systems, Inc. (Minneapolis, MN, USA). The antibodies against β-actin, MMP-1, TGF-β1, type I procollagen, elastin, p-c-Jun, and p-c-Fos were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Cell Science (Canton, MA, USA). Secondary antibodies conjugated to horseradish peroxidase were purchased from Santa Cruz Biotechnology (USA).

2.2. Sample Preparation

The dried leaves of neem (10 g) were powdered and extracted three times in 1000 ml of 50% ethanol for 24 h at room temperature. The extracts were combined, filtered, and concentrated by vacuum evaporation at 38 °C.

2.3. High-performance Liquid Chromatography (HPLC) Analysis

HPLC was performed on a Dionex Chromelon™ chromatography data system with P580 and a UVD100 detector (Thermo Fisher Scientific Inc., Waltham, MA, USA). The chromatographic column used was a Waters Sunfire C_{18} (250 × 4.6 mm, 5 μm particle size). Elution was performed using a methanol/acetonitrile (3:1) gradient containing 1% formic acid. The gradient was increased gradually from 10% to 90% methanol over 35 min. The injection volume was 10 μl, and the flow rate was 1 ml/min.

2.4. Cell Culture

NHDFs were obtained from a skin biopsy from a healthy young male donor (MCTT Bio, Inc., Seoul, Korea). NHDF cells were cultured in 100-mm dishes in DMEM supplemented with 10% heat-inactivated FBS and 1% penicillin-streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2. NHDF cells were used between passages 5 and 10.

2.5. UVB Irradiation and Sample Treatment

Cells were seeded in 35-mm tissue culture dishes. When the cells reached 80% confluence, they were subjected to irradiation supplied by a Bio-Link BLX-312 (VilberLourmatGmbH, VilberLourmat, Marnela-Vallée, France) crosslinker system with 144 mJ/cm^2, as described previously [15]. After irradiation, cells were treated with NLE (1, 10, 50, and 100 μg/mL). Control cells were exposed to normal DMEM medium without NLE.

2.6. DPPH Radical Scavenging and ROS Generation Activities

The scavenging activity of the free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) of neem leaf extract (NLE) was determined. Arbutin (0.1, 1, 10, 100, and 250 μg/mL) was used as positive control. After samples were incubated with DPPH for 30 min at 37 °C in the dark, the optical density (OD) was measured at 520 nm.

After 24 h of UVB irradiation, cells were stained with 2′7′-dichlorofluorescein diacetate (DCFH-DA; Sigma-Aldrich) for 30 min at 37 °C and then analyzed using a fluorescence microplate reader (Molecular Devices FilterMax5, USA; Excitation/Emission = 485/525 nm).

2.7. Measurement of MMP-1, IL-6, and TGF-β1 Secretion

After 72 h of treatment, the portion of supernatants was transferred into new tubes for ELISA assay. And cells were incubated with MTT and then solubilized in DMSO at the same time, as described by Hwang et al. [15]. The absorbance was determined at 570 nm using a multi-mode microplate reader (Molecular Devices FilterMax5, Sunnyvale, CA, USA). Experiments were performed using commercially available ELISA kits (MMP-1, IL-6, and TGF-β1), according to the manufacturer's instructions. Each sample was analyzed in triplicate.

2.8. Reverse Transcription (RT)-PCR

For reverse transcription-polymerase chain reaction (RT-PCR), NHDF cells were harvested 24 h after UVB irradiation. RNA was isolated from cells according to the extraction method of Hwang et al. [15]. PCR amplification was performed using a PCR premix (Bioneer) and the following primer pairs: MMP-1, forward 5′-ATT CTA CTG ATA TCG GGG CTT TGA-3′, reverse 5′-ATG TCC TTG GGG TAT CCG TGT AG-3′; type I pro-collagen, forward 5′-CTC GAG GTG GAC ACC ACC CT-3′, reverse 5′-CAG CTG GAT GGC CAC ATC GG-3′; and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), forward 5′-ACC ACA GTC CAT GCC ATC AC-3′, reverse 5′-CCA CCA CCC TGT TGC TGT AG-3′. PCR products were separated by gel electrophoresis on 2.0% agarose and visualized with ethidium bromide staining under UV illumination. GAPDH was used as an internal control. Each experiment was repeated at least three times.

2.9. Experimental Animals

Four-week-old male hairless mice (SKH: HR -1) (22–24 g; n = 20) were obtained from Central Lab Animals, Inc. (Seoul, Korea). The animals were randomly divided into five groups (designated as Normal, Cont UVB, Positive control, and Samples 1% and 5%) of four mice per cage and housed in conditions of 22 ± 1 °C, 60 ± 5% humidity, and 12 h light/dark cycles. Animals were adapted for two weeks before the study was initiated. The experimental protocol [KHU ASP (SU)-12-09] was approved by the Institutional Animal Care and Use Committee of Kyung Hee University.

2.10. UVB Irradiation in the Mouse Model

UVB irradiation and sample treatment were performed according to the method previously reported by Hwang et al. [15]. Five Sankyo Denki sunlamps with peak irradiance at 310 nm were used to perform UVB irradiation (G9T5E lamps, Sankyo Denki, Hiratsuka, Japan). Irradiance was measured using an IL1700 Research Radiometer (International Light, Inc., Newburyport, MA, USA). Bulbs were positioned 15 cm above the mice. In the topical application group, mice were exposed to 100 mJ/cm^2 UVB radiation seven times per week for the first week and then 200 mJ/cm^2 UVB radiation three times a week for four weeks thereafter [15].

2.11. Topical Application

Twenty hairless mice were divided into five groups of four mice per cage: (a) Normal (no UVB exposure; treatment with 7:3 [v/v] propylene glycol/ethanol-PGE); (b) Control (UVB exposure + treatment with PGE); (c) PC (positive control, UVB irradiation with 1% retinyl palmitate [RP] treatment); (d) NLE 1% (UVB exposure + 1% NLE treatment); and (e) NLE 5% (UVB exposure + 5% NLE treatment). During the first week, all mice were exposed to UVB without any other intervention. Thereafter, hairless mice were treated for four weeks with either vehicle (PGE) or a mixture of 1% or 5% NLE or 1% RP three times a week following 1 h of UVB irradiation. The normal group was not exposed to UVB radiation.

2.12. Histopathological Investigation

To evaluate skin wrinkles, skin replicas were made from the dorsal skin of hairless mice using SIL FLO impression material (Flexico, Colches ter, UK). A visiometer technique was used to detect changes in the transparency of the thin silicone gel prints of skin surfaces. Data were collected with a CCD video camera. The image files were analyzed using Skin Viscometer SV 600 software (Courage & Khazaka, Cologne, Germany). Skin texture was analyzed using the following roughness parameters: skin roughness (RT), maximum roughness (RM), average roughness (RZ), smoothness depth (RP), and arithmetic average roughness (RA).

Erythema index (EI) was measured with a colorimeter (Dermacatch®; Colorix, Neuchatel, Switzerland). Skin hydration was measured using a commercial contact imaging system, Epsilon (Biox Systems Ltd., England).

Skin samples were fixed in 10% neutral formolsaline, embedded in paraffin, and cut into 5 μm sections. Then, samples were stained with hematoxylin-eosin (HE) and Masson's trichrome (MT) and examined under a light microscope (Nikon Eclipse Ci attached Kameram Digital Image Analyze System) as described by Hwang et al. [15,16]. In our histopathological analyses, peritoneal adhesions, epithelial invasion between muscle fibers, cyst-like structures and their epithelial cells, vascularized granulation tissue, inflammatory cells, extra-vasated erythrocytes and hemosiderosis were scored as mild, moderate, or

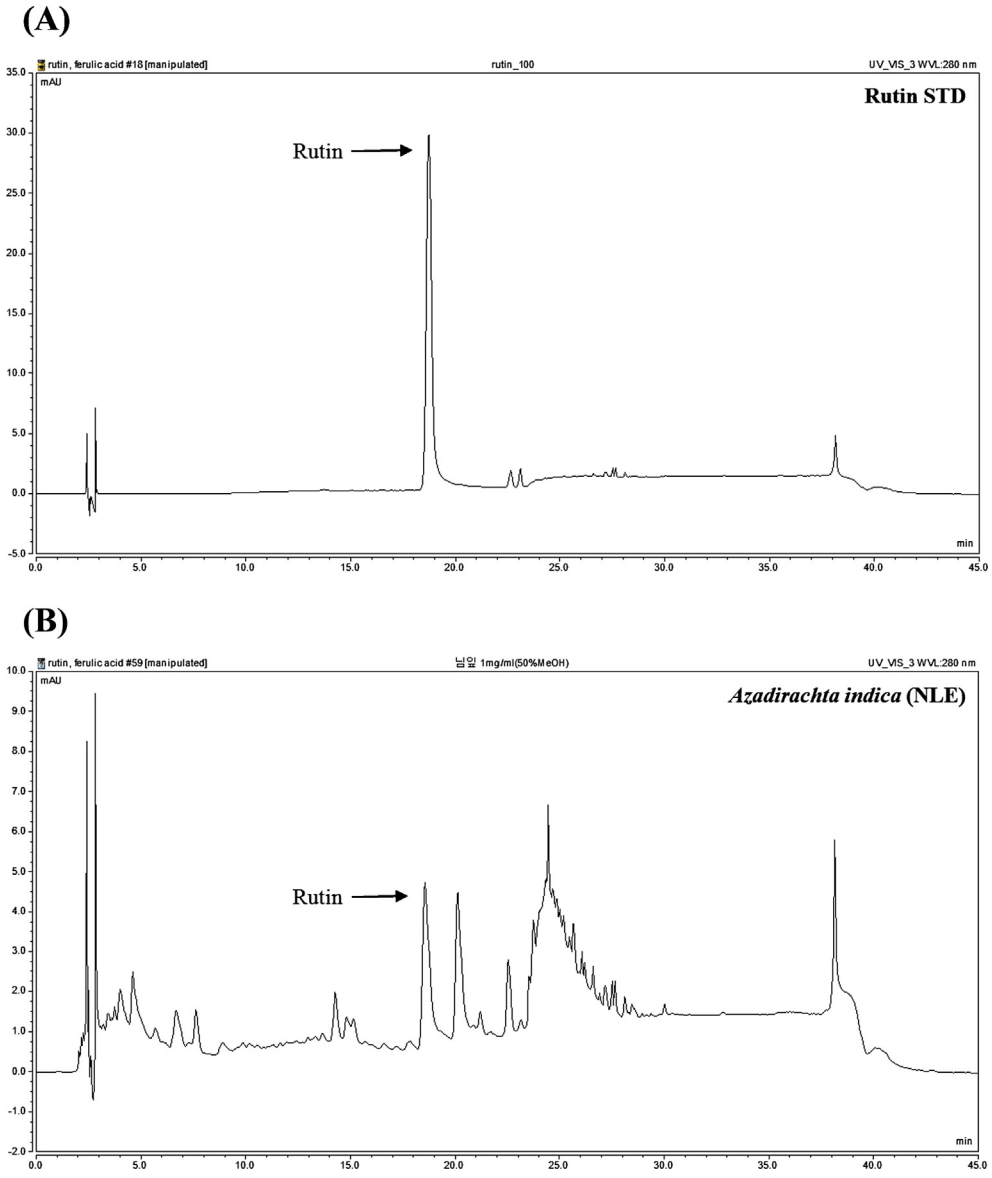

Fig. 1. LC/MS results of (a) Rutin standard; (b) Rutin extracted from neem extraction.

severe according to the severity in all specimens, and then the group averages were calculated.

2.13. Western Blot Analysis

After UVB irradiation for 4 h, NHDF cells were harvested. Skin tissues were harvested during surgeries performed on the mice on the last day of the experiment. For Western blot analysis, proteins were extracted and analyzed using methods described by Hwang et al. [15]. Protein concentration was measured using Bradford reagent (Bio-Rad, Hercules, CA, USA). Homogenized proteins were separated by sodium dodecyl sulfate–polyacrylamide gel (SDS-PAGE) and were transferred to a nitrocellulose membrane (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom). This membrane was blocked, and primary antibody was added overnight. After incubation with secondary antibody, the protein level was determined using chemiluminescence detection ECL reagents (Fujifilm, LAS-4000, Tokyo, Japan) and ImageMaster™ 17 2D Elite software, version 3.1 (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

2.14. Statistical Analysis

Statistical comparisons between different groups were performed using one-way analysis of variance (ANOVA) followed by Duncan's test. Student's *t*-test was used to compare individual treatments with controls. p-Values < 0.05 were considered significant. All data are expressed as mean ± standard deviation (SD) of three independent experiments.

3. Results

3.1. Analysis of Rutin From Neem (A. indica)

Dried neem leaves (10 g) were extracted in 50% ethyl alcohol, resulting in 29% yield of crude product (2.9 g). Rutin was identified from the ethanolic NLE. As shown in Fig. 1, peaks of NLE were identified by comparison with the retention times obtained using a rutin standard. The proportion of rutin in NLE was 1.888% (Fig. 1B).

3.2. Antioxidant Activity and Intracellular ROS Production Activity

The ability of NLE to scavenge DPPH radicals was superior to that of arbutin. In particular, NLE inhibited DPPH radicals by 82.7% at 100 μg/mL and 94.4% at 250 μg/mL (Fig. 2A). The IC_{50} values of arbutin and NLE were 195.3 and 37.3 μg/mL, respectively.

As shown in Fig. 2B, UVB-irradiated cells demonstrated a noticeably increased ROS generation compared with non-irradiated cells. NLE treatment at concentrations of 10 and 50 μg/mL led to respective 19.6% and 23.3% decreases in ROS level compared with the UVB-irradiated control cells.

3.3. MMP-1, IL-6, and TGF-β1 Expression and Cell Viability in UVB-irradiated and NLE-treated Cultured Human Dermal Fibroblasts

The viability of cells treated with UVB (144 mJ/cm^2) alone was compared to that of cells treated with UVB (144 mJ/cm^2) plus NLE

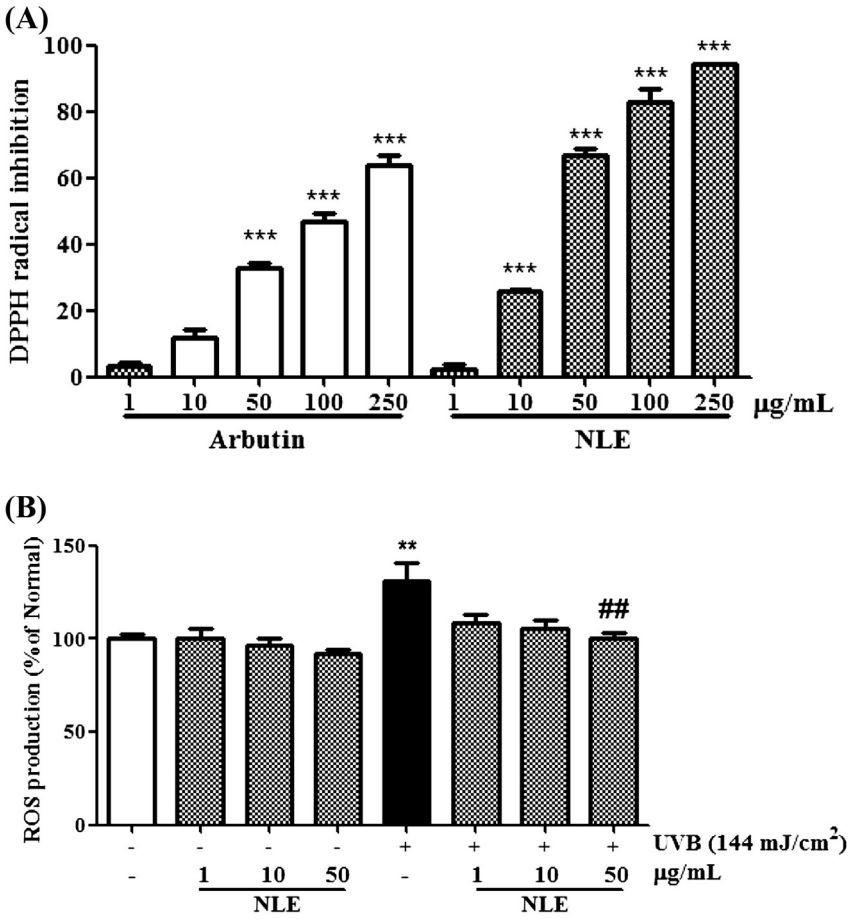

Fig. 2. Antioxidant activity and intracellular ROS production activity. In DPPH radical scavenging (A), *, **, *** indicate significant differences ($p < 0.05$, $p < 0.01$, $p < 0.001$, respectively) compared to the blank value. In ROS production (B), values shown are means ± SD. * and # indicate significant differences ($p < 0.05$) between non-irradiated control and UVB-irradiated control, respectively. **$p < 0.01$, versus non-irradiated control. ##$p < 0.01$, versus UVB-irradiated control.

(1–50 μg/mL). Non-irradiated control cells were considered to be 100% viable. The viability of cells treated with UVB radiation alone was about 80%, while that of UVB- irradiated cells treated with NLE was similar as that of control cells (Fig. 3A).

The effects of NLE on MMP-1, IL-6, and TGF-β1 expression were studied in cultured NHDFs with and without UVB exposure. In UVB-irradiated cells, MMP-1 and IL-6 secretion increased by 197.6% and 158.2%, respectively, while TGF-β1 secretion decreased by 65.3% compared with the levels in non-irradiated cells (Fig. 3B, C, and D). However, NHDFs treated with 50 μg/mL NLE had 87.9% and 77.3% lower levels of MMP-1 and IL-6 secretion, respectively, and 249.5% higher level of TGF-β1 secretion compared to cells treated only with irradiation.

3.4. Effects of NLE on MMP-1 and Type I Procollagen mRNA Expression in NHDFs

We measured MMP-1 and type I procollagen mRNA expression levels in irradiated NHDFs with and without NLE (10 and 50 μg/mL). The RT-PCR results showed weak basal expression of MMP-1 mRNA in quiescent NHDFs, which was enhanced by UVB irradiation. This elevated MMP-1 gene expression was decreased by NLE treatment in a dose-dependent manner. MMP-1 gene expression decreased by 77.2% at 10 μg/mL and 23.6% at 50 μg/mL NLE (Fig. 4A and B). In contrast, UVB irradiation markedly reduced type I procollagen mRNA expression compared with that in the control group. Type I procollagen mRNA level of cells treated with 10 μg/mL NLE was almost similar with that of UVB irradiation only. However, after 50 μg/mL NLE treatment, they were increased by 373.1% compared with that of UVB irradiation only (Fig. 4A and C).

3.5. Inhibitory Effect of NLE on AP-1 Activation

The UV-inducible transcription factor AP-1 is composed of the c-Jun and c-Fos proteins. To investigate the effects of NLE on UVB-induced AP-1 expression, human dermal fibroblasts were irradiated with UVB at 144 mJ/cm^2 and then treated with NLE for 4 h. The levels of phosphorylated c-Jun and c-Fos increased in UVB-irradiated fibroblasts. NHDFs treated with 50 μg/mL NLE had 72.8% and 66.9% lower expression of c-Jun and p-c-Jun, respectively, compared to only irradiated-cells. The respective levels of c-Fos and p-c-Fos were 73.5% and 9.8% lower after treatment with 50 mg/ml NLE (Fig. 5). It has suggested that NLE treatment has the ability to inhibit AP-1 activation in UVB-irradiated NHDFs.

3.6. Effects of Topical NLE Application on UVB-induced Skin Photoaging in Hairless Mice

The effect of NLE on wrinkle formation caused by UVB irradiation was investigated using replicas of the dorsal skin of mice (Fig. 6A and B). Skin appearances of all groups can be assessed by visual observation. It was easy to observe that the wrinkles in the control group were deep and wide as a result of UVB irradiation. In the normal group, the wrinkles were superficial and thin. In the positive control group, a reduced number of wrinkles appeared compared to the control group, thereby demonstrating the anti-wrinkle effect of retinyl palmitate used as the positive control. The wrinkles formed in the groups treated with 1% and 5% NLE were thinner and more superficial than those in other UVB-treated groups. Similarly, all of skin roughness parameters (RT, RM, RZ, RP and RA) showed the highest efficiency was NLE 1% group (vertical bar symbol) and followed by positive control group. Most of those indexes in NLE 1% group were same with that in normal group and better than positive control group. Comparison of the severity of wrinkle formation between our NLE-treated samples and our positive control demonstrated that 1% NLE produced better results with a clear and fine appearance, yielding almost the same pattern as seen in the normal group with a large improvement relative to the positive control group.

The physiological aspects of mouse dorsal skin after NLE treatment were analyzed using an analytical system. The erythema index (EI) increased in UVB-exposed skin compared with non-exposed skin (Fig. 6C). However, treatment with NLE reversed the tendencies seen following UVB exposure alone. The groups treated 1% and 5% NLE had EI levels that decreased by 16.3% and 14.5%, respectively, compared with the control group. The levels of skin hydration are shown in Fig. 6D. The bright of green became more yellow and brighter when the skin

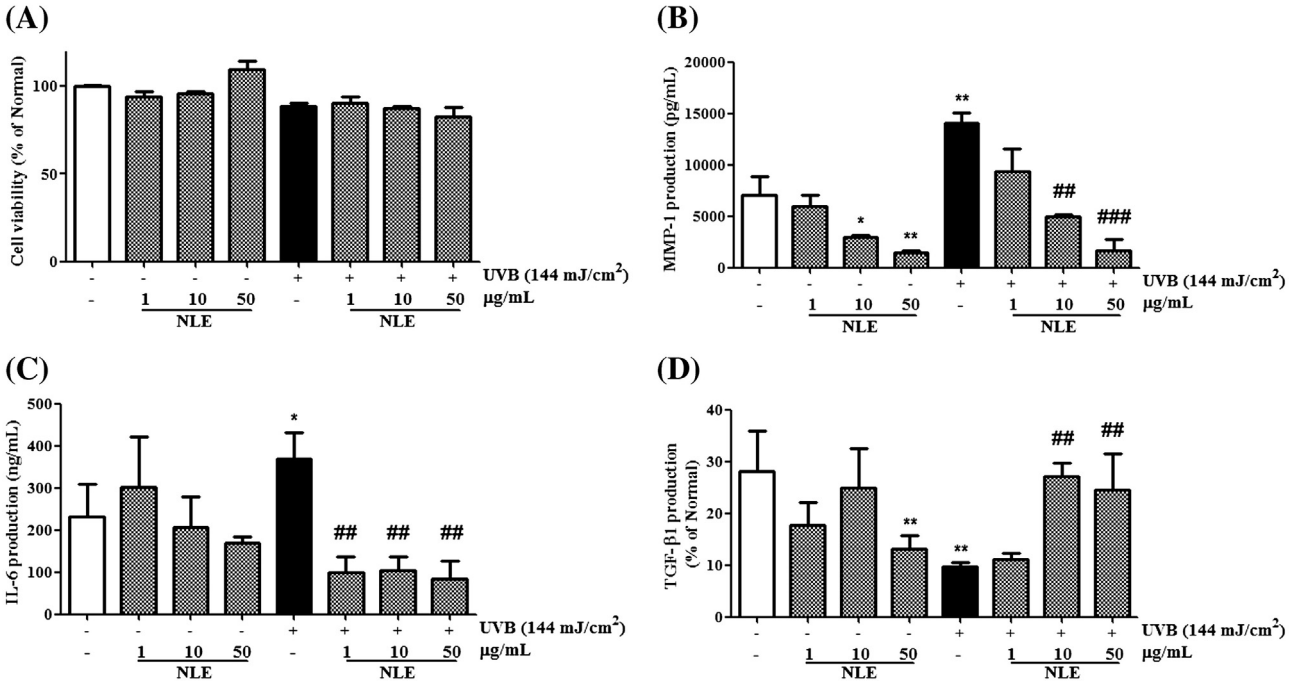

Fig. 3. Cell viability (A), MMP-1 secretion (B), IL-6 secretion (C), and TGF-β1 expression (D) in UVB-irradiated and NLE-treated NHDFs. Values are means ± SD. * and # indicate significant differences ($p < 0.05$) between non-irradiated control and UVB-irradiated control, respectively. *$p < 0.05$, **$p < 0.01$, versus non-irradiated control. ##$p < 0.01$, ###$p < 0.001$, versus UVB-irradiated control.

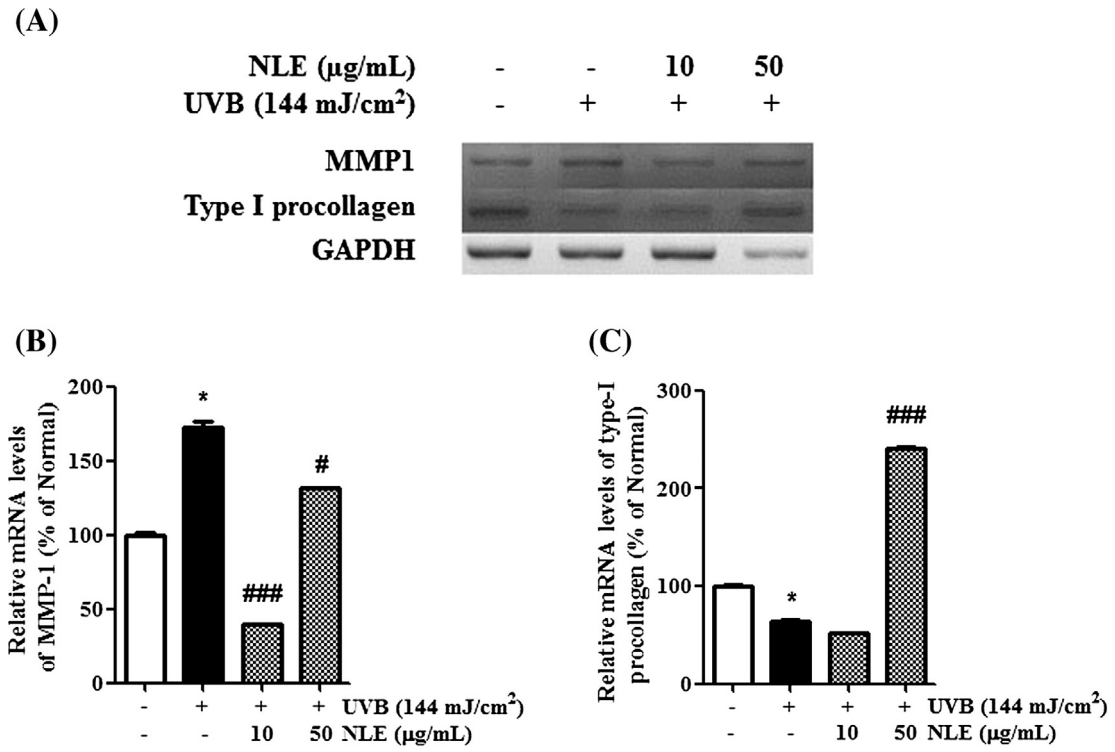

Fig. 4. Effects of NLE on MMP-1 and type I procollagen mRNA expression in NHDFs. GAPDH mRNA was used as an internal control. The bands intensities (A) were quantified by densitometry, normalized to the level of GAPDH mRNA, and calculated as the percentage of the basal response (B, C). All data are shown as the mean ± SD of at least three independent experiments performed in triplicate. $^{*}p < 0.05$, versus non-irradiated control. $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$, versus UVB-irradiated control.

hydration level increased. Compared with the UVB control group, the groups treated with positive control and NLE had significantly increased skin hydration. However, same with the case of replica analysis, NLE 5% group showed the increased wrinkle indexes and the decreased skin hydration level compared with NLE 1% group. Thus, we suggested that a better concentration should be used as 1% of NLE.

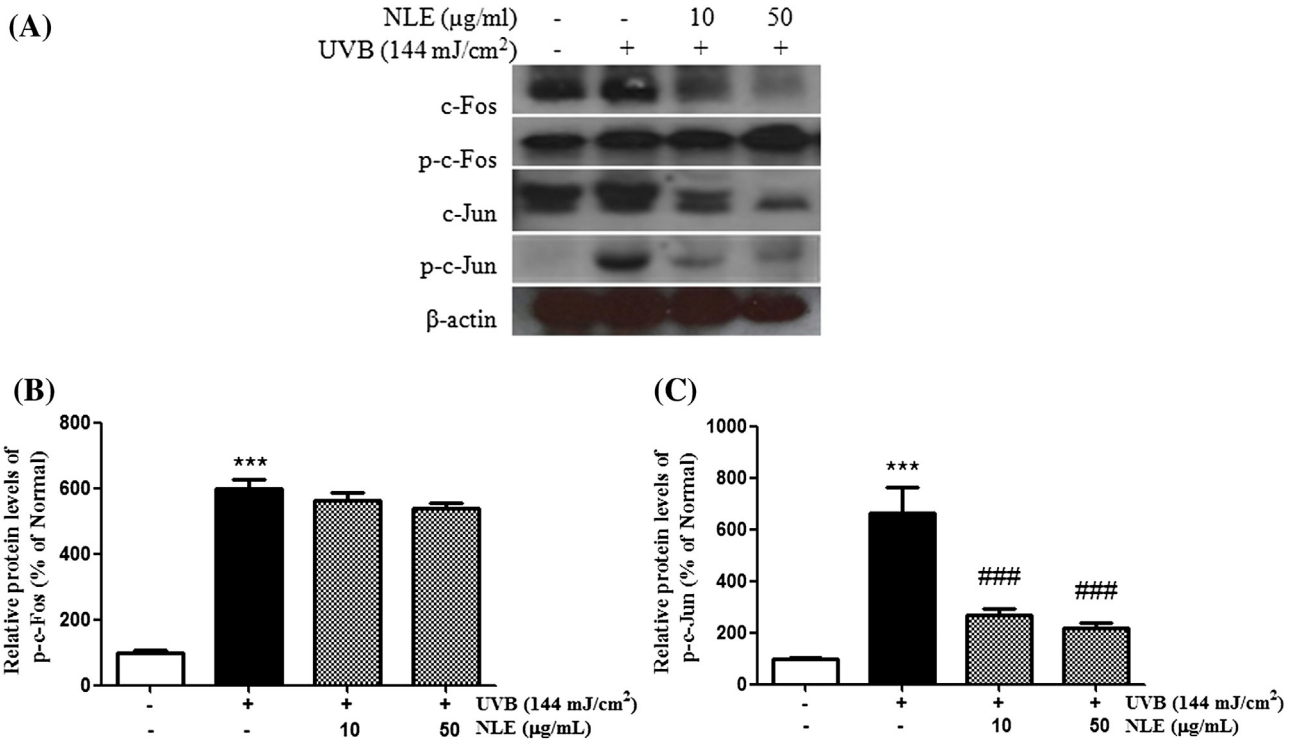

Fig. 5. Effects of NLE on AP-1 activation in UVB-exposed NHDFs. Phosphorylation of c-Fos and c-Jun was detected by Western blotting (A). β-Actin was used as an internal control. The bands intensities were quantified by densitometry, normalized to the level of actin, and calculated as the percentage of the basal response (B, C). The results are shown as the mean ± SD of at least three independent experiments performed in triplicate. $^{***}p < 0.001$, versus non-irradiated control. $^{\#\#\#}p < 0.001$, versus UVB-irradiated control.

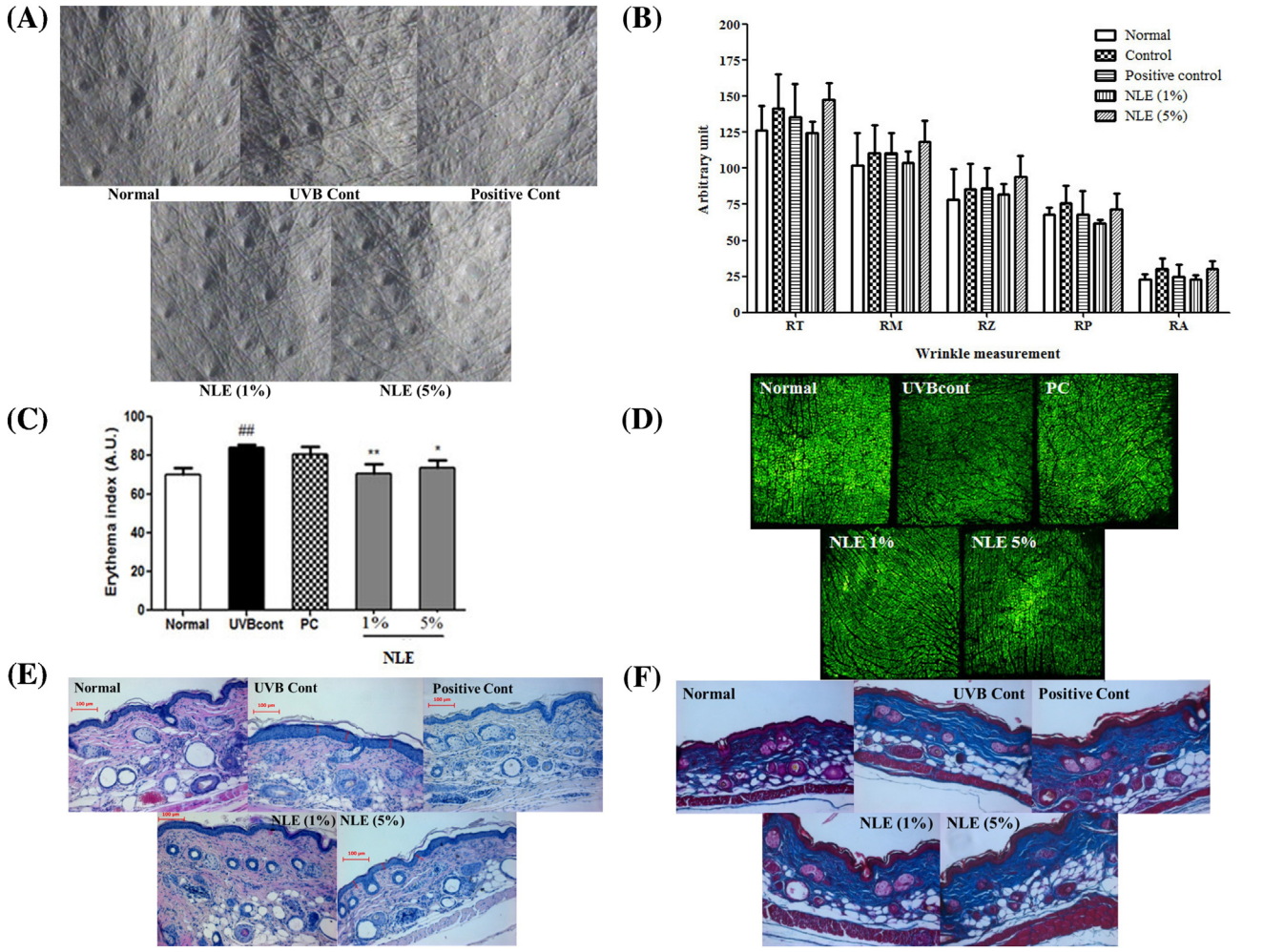

Fig. 6. Photographs of replicas (A), replica analysis (B), EI analysis (C), skin hydration (D), H&E-stained sections (E), and Masson's trichrome-stained section (F) of the dorsal skin of mice in the topical application groups. Values are means ± SD (n = 4). Data were evaluated for statistical significance with one-way analysis of variance followed by Duncan's multiple range tests. Means with the same letters are not significantly different. # and * indicate significant differences ($p < 0.05$) between non-irradiated control and UVB-irradiated control, respectively. ##$p < 0.01$, versus non-irradiated control. *$p < 0.05$, **$p < 0.01$, versus UVB-irradiated control. Red scale bar, 100 μm. PC: positive control. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

H&E staining and Masson's trichrome staining were used to evaluate the effects of NLE on histological changes in the dorsal skin. Significant histological changes, such as thickening and disruption of collagen fibers, were observed in UV-irradiated skin. However, the epidermis of the NLE group was thinner than that of the control group (Fig. 6E). Also, compared with the UVB control group, the NLE group exhibited an increased abundance and density of collagen fibers (Fig. 6F), even more so than seen in the positive control group.

UVB irradiation causes up-regulation of MMP-1 and down-regulation of elastin, type I procollagen, and TGF-β1 proteins (Fig. 7). Production of MMP-1 decreased by 21.6% and 25.7% in the groups treated with topical application of 1% and 5% NLE, respectively, compared with the control group; this was almost the same pattern we observed in the positive control group where MMP-1 was decreased by 33.0%. As a positive control, we evaluated the expression levels of type I procollagen and TGF-β1 in retinyl palmitate-treated animals and found increase of 339.6% and 137.2%, respectively; however, elastin level decreased slightly by 6.2% compared with that of the control group. In contrast, the expression levels of type I procollagen, TGF-β1, and elastin in the 1% and 5% NLE groups increased by 359.0%, 131.7%, and 136.6%; and 268.9%, 149.3%, and 161.1%, respectively, compared with levels in the control group (Fig. 7). Consequently, as demonstrated by our data on skin appearance and skin aging-related signaling molecules, NLE 1% group was the most effective group, than was the positive control of 1% retinyl palmitate, resulting in increased collagen and elastin fibers and smoother and thinner skin.

3.7. Discussion

Neem leaves are gaining interest all over the world because of their healing properties, including antioxidant, immune-modulatory, anti-inflammatory, anti-hyperglycemic, antiulcer, antifungal, antibacterial, anti-mutagenic and anti-cancer properties [7,8,19,20]. Additionally, neem was recently suggested for use as a skin whitening agent because the extract of neem bark and leaves has shown significant tyrosinase inhibition activity [21,22]. Among several methods of extraction, ethanolic neem extract from dried leaves was reported to contain high contents of flavonoids, saponins, and alkaloids, along with low levels of lipids [23–25]. Asobara et al. [26] also showed that the amounts of these constituents were greater in neem leaves were in other parts of neem. Consequently, we chose to evaluate neem leaves in skin anti-aging assays in the present study. Our free radical scavenging activity of NLE was an IC_{50} of 37.3 μg/mL, and was much lower than 50% ethanolic neem leaf extract described by Pandey et al. [27] to have an IC_{50} of 110.36 μg/mL. This finding demonstrates the increased antioxidant activity in our NLE sample.

Recently, rutin and other polyphenol compounds have been receiving considerable attention because they possess numerous

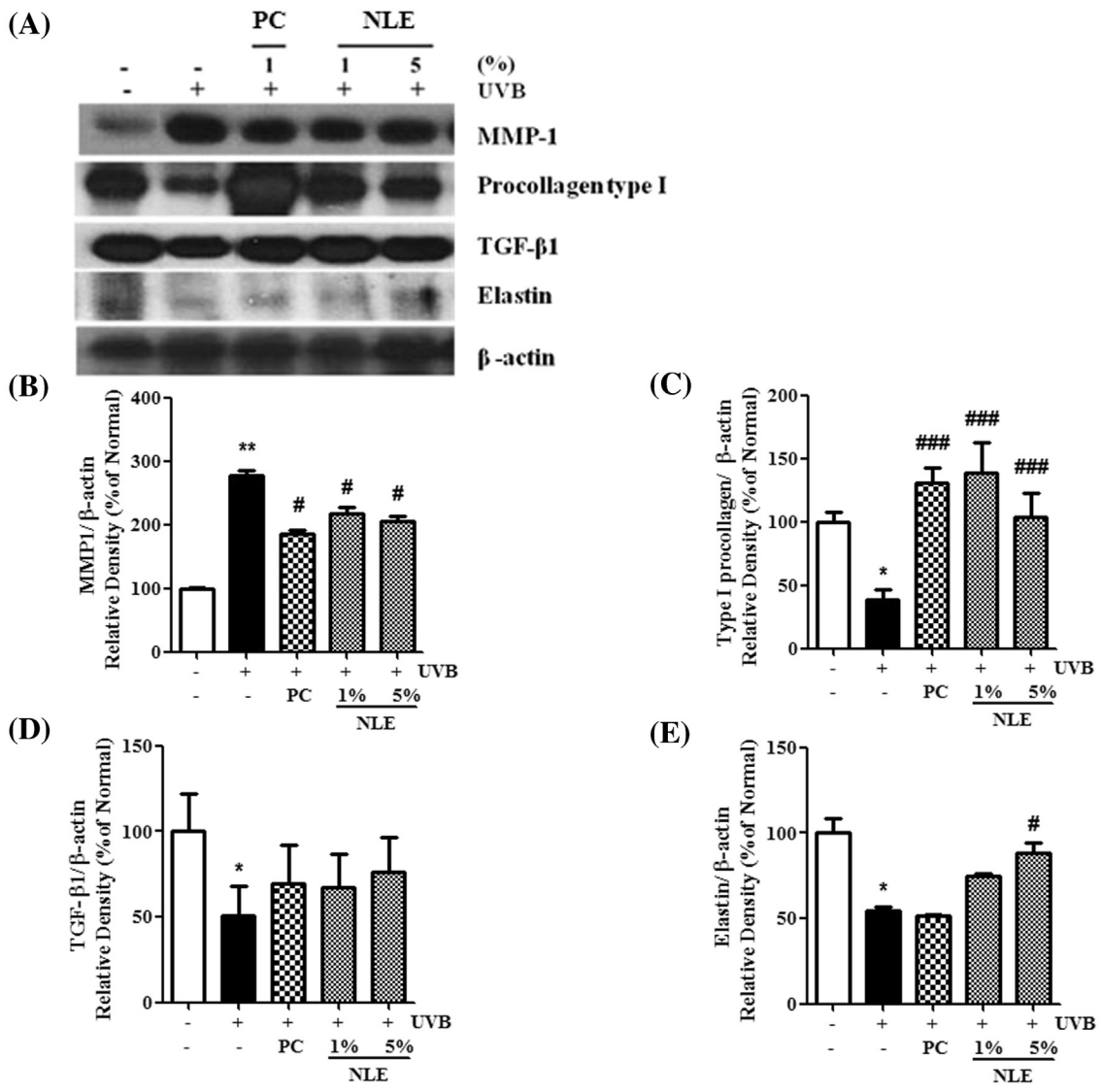

Fig. 7. Effects of NLE on MMP-1 secretion, Type I procollagen production, TGF-β1 secretion, and elastin production in UVB-irradiated and NLE-treated mice tissues (A). The bands intensities were quantified by densitometry, normalized to the level of actin, and calculated as the percentage of the basal response (B–E). Values are means ± SD. # and * indicate significant differences ($p < 0.05$) between non-irradiated control and UVB-irradiated control, respectively. *$p < 0.05$, **$p < 0.01$, versus non-irradiated control. #$p < 0.05$, ###$p < 0.001$, versus UVB-irradiated control. PC: positive control.

pharmacological properties, particularly antioxidant and anti-aging effects [28]. Jadoon et al. [29] reviewed the anti-photoaging effect of cream from some herbal extracts which contained rutin such as *Moringa oleifera* and *Ocimum basilicum*. The flavonoid rutin (quercetin-3-rhamnosyl glucoside) has been reported to be the main active constituents in neem leaves [27,30]. This major active constituent has been found to inhibit the generation of ROS, which can be activated by UV radiation [31–35]. Furthermore, rutin has been shown to possess potent anti-aging activities in mice, which are mediated through anti-oxidative mechanisms [16,32,36,37].

As our previous study, cells treated with 10 μg/mL rutin had 46.9% lower level of MMP-1 secretion compared to cells treated only with irradiation [16]. In present study, NHDFs treated with 50 μg/mL NLE (containing 0.944 μg/mL of rutin) had 87.9% lower MMP-1 level compared to cells treated only with irradiation. Although NLE treatment at 50 μg/mL obtained a smaller concentration of rutin, the ability of MMP-1 level inhibition was better. Thus, NLE composition has not only rutin but also other potential phenolic acids and they worked together to get better efficiency of NLE. Additionally, many water-soluble derivatives of rutin were shown with its greater antioxidant activity than rutin [38,39]. However, rutin still can be considered as an active compound in NLE for skin anti-aging properties.

Furthermore, Peres et al. [40] reported the anti-UVB effect of rutin in UVB filters in dermocosmetic preparations. Rutin has been determined as a potential sunscreen agent as described by Choquenet et al. [41]. Thus, in addition to many traditional treatments of skin diseases, NLE might possess the potential effect in topical application. In addition, Biu et al. [42] reported the dose-dependent toxicity of neem leaf aqueous extract in chicken, with lethal dose (LD_{50}) of 4800 mg/kg. In addition, oral administration of neem seed oil was also shown to be toxic, even though it has been used to treat some chronic skin diseases [43,44]. Based on the above information regarding the toxicity of neem, only topical application was performed in our study. As shown in Figs. 6 and 7, NLE was recommended with use concentration as 1% (not over 5%). Although NLE 5% group had no significant change in replica analysis, they had better efficiency in skin hydration, EI, thickening, and the higher levels of TGF-β1, procollagen, and elastin compared with those in control group.

On the other hand, our results showed that NLE had potential anti-wrinkle activity; wrinkles represent the most important damage

induced by UVB. The anti-wrinkle activity was clearly demonstrated by our replica, and H&E and Masson's trichrome staining data. NLE 1% group had all wrinkle indexes lower than that in control group. In Fig. 6E, it was observed that the thicker epidermis and the loss of skin natural elasticity were extremely happened in control group. NLE-treated skin tissue had thinner epidermis and integrated collagen and elastin fibers, almost the same as in our normal group skin tissues. In addition, TGF-β, a ubiquitous cytokine, also plays a key role in the syntheses of procollagen and tropoelastin (precursors of collagen and elastin, respectively) [15,45]. Loss of organization of collagen and elastin bundles in skin connective tissue is responsible for wrinkle formation in photodamaged human skin [9]. Thus, up-regulation of type I procollagen and elastin production by NLE treatment was suggested to occur through enhance TGF-β activities. Thus NLE treatment increased skin strength and resilience even after UVB exposure.

4. Conclusion

In summary, our data suggest that NLE has a strong protective effect against UVB damage in NHDFs in vitro and in an in vivo skin model through the prevention of collagen and elastin degradation. All of the effects were partly due to rutin, an active compound in NLE. This is accomplished through decreased ROS activation and MMP-1 production, as well as collagen synthesis enhancements including increased TGF-β1, type I procollagen and elastin. Moreover, our topical application data indicated that NLE has a protective effect in UVB-irradiated hairless mice by preventing wrinkle formation, skin dryness, skin erythema, and skin thickening. Thus, NLE may be potentially useful in cosmetic products, particularly in skin-care products, intended to prevent the skin damage associated with UVB-induced photoaging.

Conflicts of interest

The authors have no conflicts of interest to declare.

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