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ORIGINAL ARTICLE

Anti-aging, Anti-Inflammatory, and Wound-Healing Activities of Edible Bird's Nest in Human Skin Keratinocytes and Fibroblasts

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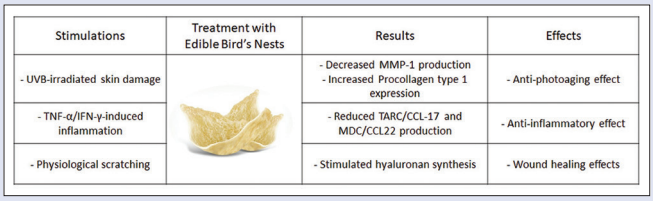
ABSTRACT

Background: The "bird's nest soup" is a kind of luxury or tonic food prepared from edible bird's nest (EBN), which is used as an extremely nutritious medicine to improve health. However, its skin anti-inflammatory and wound-healing effects are still not fully understood. **Aim:** In this study, the skin-protective effects of two geographical types of EBN (EBN-A and EBN-B) were investigated. **Materials and Methods:** The anti-aging effect was assessed in ultraviolet B (UVB)-irradiated normal human dermal fibroblasts (NHDFs), while the anti-inflammatory effect was evaluated in tumor necrosis factor alpha/interferon gamma (TNF-α/IFN-γ)-stimulated human skin keratinocytes (HaCaTs). The wound-healing activity was investigated in scratched NHDFs and hyalorunan production was examined in HaCaTs. **Results:** In this study, EBN showed good efficiency in scavenging free radicals. EBN notably decreased the UVB-induced matrix metalloproteinase-1 expression and promoted procollagen type I synthesis that resulted in the protective effect of EBN against UVB-induced skin damage. Overexpression of thymus- and activation-regulated chemokine and macrophage-derived chemokine induced by TNF-α/IFN-γ was significantly decreased after treatment with EBN at 1–10 μg/ml. The most effective wound healer was EBN-B at 1–10 μg/ml, based on the high expression of hyaluronan that has long been associated with the remodeling extracellular matrix in wound healing. **Conclusion:** These results indicate that EBNs have the potential to ameliorate UVB-induced skin photo aging and NF-α/IFN-γ-stimulated inflammation as well as wound injuries, resulting in rapid healing effects.

Key words: Bird's nest, fibroblasts, inflammation, keratinocytes, wound healing

SUMMARY

- EBN possesses the ability to alleviate ultraviolet B-induced skin damage through regulation of matrix metalloproteinase-1/procollagen type I expression. EBN blocked the overexpression of thymus-and activation-regulated chemokine/CCL-17 and macrophage-derived chemokine/CCL22 in tumor necrosis factor alpha/interferon gamma-induced inflammatory keratinocytes (HaCaTs). One of the most important effects investigated was that the wound-healing process could be significantly supported by treatment with EBN by promoting hyaluronan induction. EBN is thus a valuable product that represents a complementary and alternative medicine for skin disease treatment.



Abbreviations used: EBN: Edible bird's nest; (A-Java; B-Sumatra/Banka); NHDFs: Normal human dermal fibroblasts; HaCaTs: Keratinocytes; EGF: Epidermal growth factor; TNF-α: Tumor necrosis factor alpha; IFN-γ: Interferon gamma; MMP-1: Matrix metalloproteinase-1; TARC: Thymus-and activation-regulated chemokine; MDC: Macrophage-derived chemokine.

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INTRODUCTION

Edible bird's nest (EBN; cubilose) is a nest prepared by the salivary secretion of specific swiftlets during their breeding stage, for example, the genera *Aerodramus* and *Collocalia*. Because it is well-known to be rich in nutrients, EBN has been used both as a delicacy and a pharmaceutical only by Asian royalty since the ancient times. Three decades ago, the beneficial properties of EBN were proven using modern scientific techniques, which revealed its various pharmacological activities, including antioxidative effects,[1] anti-inflammatory effects,[2] anti-influenza effects[3] and skin whitening effects,[4,5] along with promotion of corneal wound healing,[6] improvement of stem cell proliferation,[7] memory improvement and neuroprotection in Alzheimer's or Parkinson's Disease,[8-10] epidermal growth enhancement,[11] osteoporosis-improving,[12] antiobesity effects,[13] and prevention of cardiometabolic and diabetic diseases.[14]

EBN comprises 40%–60% proteins, 10%–30% carbohydrates and 6%–13% sialic acid and has more than 78 detected metabolites, including amino acids like aspartic acid, arginine, histidine, leucine, glutamic acid, proline, serine, threonine and valine and minerals such as Na, Ca, Mg, K, Al, and Sr.[15-19] Sialic acid is extremely important to stabilize the nest and is distributed evenly inside the nest. The major sialic structure detected in EBN was N-acetyl-neuraminic acid (up to 99% of total sialic acid content).[20] Mehdi *et al.* showed that oxidative stress during aging can affect sialic acid decomposition from the membrane and that sialic acid can prevent these processes.[21] However, it is still difficult to identify the proteomic profile of EBN due to its poor solubility and low extractive rate. The proteins in EBN are often extracted using distilled water heated to 60°C–100°C.[22]

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Skin is the primary barrier against external damage; however, extrinsic factors such as UV lights, wounds, or inflammatory inducers can damage the skin leading to dryness, pigmentation, epidermal thickening and wrinkling with delayed recovery of skin health.[23] UV is the major factor that destroys skin and ultraviolet B (UVB) can reach the dermis layer of skin where fibroblasts are located and cause serious cellular oxidative stress. This reduction of oxygen balance induces skin inflammation and photoaging. Skin photoaging was suggested to occur due to the degradation of collagen, a primary constituent of the extracellular matrix, by overexpression of matrix metalloproteinases (MMPs), notably MMP-1.

The second factor causing skin damage includes scratch wounds that are frequently formed in daily life, especially in children. Based on their size and depth, the severity of skin wounds can be predicted to have quick or slow recovery. Often, the epidermis and the dermis layers are easily affected by skin injuries and the recovery of these cells can decide how fast wound healing occurs. Wound closure and re-epithelialization can be supported by fibroblast migration. In a variety of healing factors such as age, sex hormones, diabetes, obesity, infection, stress, alcoholism, smoking and so on, nutrition plays a key role with well-known effects by both oral and topical therapies.[24] Recently, scientists rediscovered hyaluronan as a major player in wound healing.[25,26] This secreted protein of HaCaTs keep skin moisturized and activates HaCaT migration resulting in quicker healing.

Finally, inflammatory mediators can be considered as toxic chemicals to skin cells. Tumor necrosis factor-α (TNF-α), interferon-gamma (IFN-γ), or interleukins (ILs) activate the inflammation process in HaCaTs and cause its apoptosis. At high severity levels of inflammation, HaCaTs produce chemokines such as thymus-and activation-regulated chemokine (TARC) and macrophage-derived chemokine (MDC) to regulate intercellular communication. Thus, limited expression of these cytokines can contribute to reverse chronic inflammation in the skin. Nowadays, skin strength, resilience and rapid recovery can be facilitated by various herbal medicines. Based on the history of EBN use, it is hypothesized to have good skin protective effects. Up to date, it has not yet clarified the role of EBN in wound healing. In this study, the effects of EBN water extracts on UVB-and scratch treated fibroblasts (NHDFs) and TNF-α/IFN-γ-induced HaCaTs was investigated through the collagen/MMP-1 relationship and the expression of hyaluronan and chemokines.

MATERIALS AND METHODS

Extraction method

EBNs were purchased from markets in Indonesia. Dried EBNs were extracted in 500 mL of water for 3 days at 37°C in a thermostatic chamber, filtered and concentrated at 40°C. The extracts A (EBN-A, collected from Java city) and B (EBN-B, collected from Sumatra/Banka cities) were lyophilized and stored at −20°C.

High-performance liquid chromatography analysis

Sialic acid was identified according to the uses of ultra performance liquid chromatography-UPLC (Acquity UPLC, Waters, Milford Massachusetts, USA) and Quadrupole mass (Xevo TQ-S micro, Waters). The Imtakt Unison UK-Amino C_{18} column (Supleco, 100 mm × 2 mm, 3 μm) was used at 30°C. A 2 μL sample was injected at a flow rate of 0.3 mL/min. Mobile phase was used with 10 mM ammonium formate: Acetonitirile. Sialic acid was detected at m/z Q1: 308.20 → Q3: 86.98 CE: 30.

The epidermal growth factor (EGF) standards were prepared in 5% of sample with the high-performance liquid chromatography system (Agilent 1100 LC system, Agilent, Seoul, Korea). A μBondapak C_{18} column 125 Å (0.39 × 300 mm) was used to detect the EGF content of EBN. Other setting conditions were a 10 μL sample was injected at a flow rate of 1 mL/min. Mobile phase was used with distilled water: 0.1% trifluoroacetic acid in acetonitrile.

Cell culture

HaCaTs, originating from human HaCaTs were purchased from the Korea cell line bank (Seoul, Korea). Normal human dermal fibroblasts (NHDFs) were purchased from Sciencell Research Laboratories (California, USA). Cells were cultured in DMEM supplemented with 10% heat-inactivated FBS and 1% penicillin–streptomycin (Gibco BRL, Grand Island, NY, USA). Cell cultures were maintained in a humidified incubator with 5% CO_2 at 37°C. HaCaT and NHDF cells were seeded at cell densities of 1×10^4 and 2×10^4 cells/cm^2. Cells are ready for experiments when it reaches 80% confluence.

Cell viability assay

The MTT assay was used to detect cell viability. NHDFs and HaCaTs in the logarithmic phase were counted and seeded in 96-well plate. After 24 h treatment with EBNs (0.01, 0.1, 1 and 10 μg/mL) or UVB radiation or other factors, 0.1 mg/mL of methylthiazolyldiphenyl-tetrazolium bromide (MTT, Sigma-Aldrich, Korea) was added and incubated for 3 h. Dimethyl sulfoxide (DMSO) was used to dissolve the formazan crystals. The absorbance was read at 570 nm using a micro plate reader (Molecular Devices E09090; San Francisco, CA, USA). The experiment was repeated six times, independently.

Scratching assay

The wound-healing migration rates of NHDFs were assessed by the scratch assay method. Before seeding, the wound field inserts (Cell Biolabs, Seoul, Korea) were warmed up and contacted with the bottom of the plate well. The cells were seeded at 8×10^4 cells/cm^2 into a 24-well plate. After 24 h of incubation, the 90%–100% confluent cells can firmly reach. The inserts were removed carefully from the well to begin the wound-healing assay. The debris was removed by washing with PBS. The cells were treated with EBN extracts at a range of concentrations (0.01–10 μg/ml) in serum-free media. Allantoin (10 μg/ml) was used as a positive control. The scratch, which represented a wound, was photographed at 0, 6, 12, 18 and 24 h using phase contrast microscopy at ×10 magnification. The images were analyzed using ImageJ software to determine the wound-healing rate (% of control). Experiments were performed in the triplicate manner.

Measurement of hyaluronan secretion

DMSO was served as a vehicle. Secretion of hyaluronan in HaCaT cell culture supernatants was determined using a Hyaluronan DuoSet ELISA kit (R and D Systems, MN, USA) following the manufacturer's instructions. In brief, the plate preparation was sealed with capture reagent overnight and blocked by 300 μL of reagent diluent for 1 h at room temperature. Samples or standards in reagent diluent were added and incubated for 2 h. Hyaluronan concentration was measured by using streptavidin-HRP B-combined detection reagent and substrate/stop solution. The absorbance wavelength was set from 450 to 540 nm.

Ultraviolet B irradiation and sample treatment

The UVB source (Bio-Link BLX-312, Vilber Lourmat, Gmbh, France) had a spectral emission at 312 nm. Cells were subjected to a UVB dose of 144 mJ/cm^2, and UVB irradiation lasted 65 s.

Tumor necrosis factor alpha/interferon gamma-induced inflammatory keratinocytes

The inhibitory effects of EBN extracts were evaluated in TNF-α/IFN-γ-stimulated HaCaTs. Cells were seeded in 96-well culture plates and incubated for 24 h at 37°C in a humidified atmosphere containing 5% CO_2. The cells were pretreated with the extracts (0.01–10 μg/ml μg/ml) for 30 min and then treated with 10 ng/mL of TNF-α/IFN-γ for next 24 h in serum-free media. At the same time, a cell viability assay was performed to assess the cellular cytotoxic effect.

Messenger RNA expression analysis by reverse transcription-polymerase chain reaction (RT-PCR)

Following UVB (144 mJ/cm^2) irradiation and TNF-α/IFN-γ (10 ng/mL) treatment for 24 h, RNA isolation was performed using the TRIZOL reagent (Invitrogen Life Technologies, Carlsbad, CA). Polymerase chain reaction (PCR) amplification was performed by PCR premix (Bioneer CO., Korea). Reverse transcription-PCR (RT-PCR) was performed in a Veriti Thermal Cycler (Applied Biosystems, Foster City, CA, USA) and 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'; Procollagen type I, forward 5'-CTC GAG GTG GAC ACC ACC CT-3', reverse 5'-CAG CTG GAT GGC CAC ATC GG-3'; TARC/CCL17, forward 5'-ATG GCC CCA CTG AAG ATG CT-3', reverse 5'-TGA ACA CCA ACG GTG GAG GT-3'; MDC/CCL22, forward 5'-AGG ACA GAG CAT GGC TCG CCT ACA GA-3', reverse 5'-TAA TGG CAG GGA GGT AGG GCT CCT GA-3'; and human GAPDH, forward 5'-ACC ACA GTC CAT GCC ATC AC-3', reverse 5'-CCA CCA CCC TGT TGC TGT AG-3'). PCR was performed using a Veriti Thermal Cycler (Applied Biosystems, Foster City, CA, USA). PCR products were separated by 2.0% agarose gel electrophoresis and visualized with ethidium bromide staining. The experiment was repeated three times, independently.

Statistical analysis

Value was expressed as mean ± standard deviation by the GraphPad Prism 5 (GraphPad Software, Inc., CA, USA). Student's *t*-tests were performed to compare individual treatment to the control and comparisons between different treatments were analyzed by one-way analysis of variance with a significance level of $P < 0.05$. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ were considered statistically significant compared with the basal cells; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ were considered statistically significant compared with only UVB-irradiated cells or only TNF-α/IFN-γ-treated cells.

RESULTS

Sialic acid and epidermal growth factor contents of edible bird's nest

Total sialic acid content of EBN was 13,084.3 ng/mL for EBN-A and 29,131.1 ng/mL for EBN-B [Figure 1]. The EBN extracts were rich of EGF component with 31,823 ng/mL in EBN-A and 43,885 ng/mL in EBN-B [Figure 2].

Wound-healing effect of edible bird's nests on fibroblasts and keratinocytes

The effect of EBN extracts on cell viability was investigated in NHDF and HaCaT cells by MTT assay. As shown in Figure 3, the viabilities of cells were not significantly decreased by treatment with both EBN extracts at 0.01–10 μg/mL compared to non-treated cells.

Fibroblasts can be self-healed by 17.0% without any treatment after 24 h. However, the wound-healing rate was significantly increased by 28.2% in the allantoin (10 μg/mL)-treated cells compared to 0 h point time-recorded cells. Notably, treatments with EBN-B at 10 μg/mL led to healing rates (almost diminishing the wound area) better than that seen in the positive control group by 39.6%.

The production of hyaluronan, an important factor of wound-healing process, was measured in HaCaTs. The production of hyaluronan was significantly elevated by 373.9% using retinoic acid as a positive control. The extracts of EBN was slightly increased the hyaluronan by 109.1% at 10 μg/mL of EBN-B.

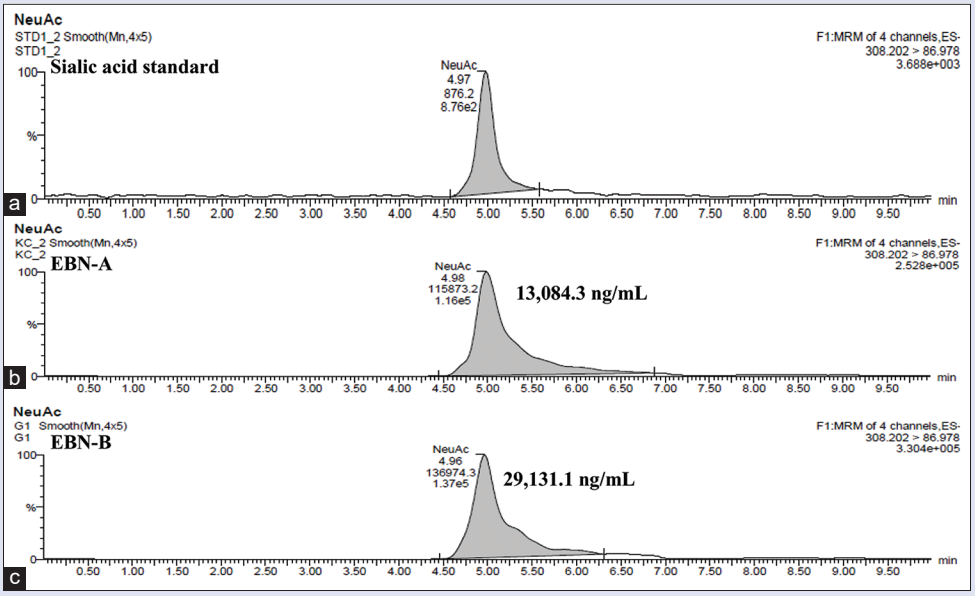

Figure 1: High-performance liquid chromatography analysis of sialic acid from edible bird's nest extracts. (a) Sialic acid standard; (b) Edible bird's nest-A; (c) Edible bird's nest-B

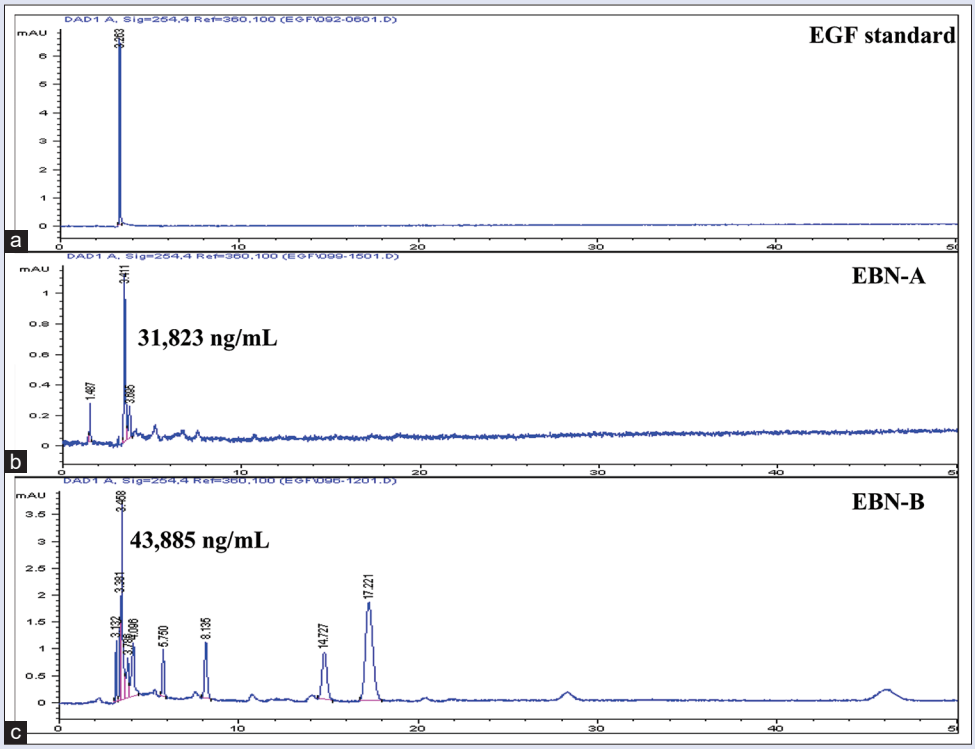

Figure 2: High-performance liquid chromatography analysis of epidermal growth factor, epidermal growth factor from Edible bird's nest extracts. (a) Epidermal growth factor standard; (b) Edible bird's nest-A; (c) Edible bird's nest-B

Cell viability and matrix metalloproteinase-1/procollagen type I messenger RNA expression in ultraviolet B-irradiated normal human dermal fibroblasts

Following UVB irradiation (144 mJ/cm^2), NHDFs were treated with EBN (0.01–10 μg/mL) for 24 h showed the non-significance of cellular cytotoxic effects. As shown in Figure 4, UVB irradiation led to an increase of mRNA MMP-1 production by 117.8% and a decrease of 13.8% mRNA procollagen type I expression. However, the presence of EBNs, particular EBN-B, significantly diminished MMP-1 expression around 29%–70% and enhanced procollagen type I expression from 158 to 243%, compared with UVB-irradiated cells.

Effects of Edible bird's nest on tumor necrosis factor alpha/interferon gamma-induced inflammatory keratinocytes

The study was designed to keep no-effect on cell viability under conditioning stimulation with TNF-α/IFN-γ. In spite of that, TNF-α/IFN-γ significantly caused the high expression of inflammatory chemokines including TARC/CCL17 and MDC/CCL22. As respected, these overexpression was effectively decreased by 89.7% for TARC and 46.1% for MDC after treatment with EBN-B at 10 μg/ml [Figure 4].

DISCUSSION

In this study, the anti-aging, anti-inflammatory and wound-healing effect of EBN extracts were investigated with EBNs collected from two different locations. EBN-B, collected at Sumatra/Banka cities displayed better skin protective effects compared to EBN-A, collected at Java city. Similar to Vimala *et al*. different samples of the raw EBN from geographical locations such as the North, South, or East Coast zones of Peninsular Malaysia were reported to have different anti-inflammatory activities.[2] Additionally, EBNs from Peninsular and East Malaysia have been shown to result in the development of different nutritional, physicochemical and antioxidant databases.[27] EBNs from different parts of Peninsular Malaysia or East Malaysia, or from heavily polluted industrial areas have revealed that the geographical factor has an important effect on their neural anti-inflammatory effect in cognitive disorders.[28] In addition to EBN, the propolis collected by honeybees from plants in different geographic locations (different cities of Indonesia, Iran, Brazil, Cuba, Portugal) can also have different *in vitro* antiplasmodial activities.[28] Many plant extracts from different collection sources have also demonstrated different biological activities.[29-31]

As mentioned in the Introduction, EBN compositions are diverse and known to include proteins, carbohydrates, sialic acid and amino acids. Although the specific bioactive compounds accounting for the pharmacological effects observed are yet to be identified in many studies, the complex compositional makeup of EBN may suggest a plethora of therapeutic potentials. Based on experience with many detections, sialic acid and EGF contents were highly identified in EBN extracts [Figures 1 and 2]. Sialic acid is mainly derived from mammalian sources such as human milk and skin and from royal jelly.[32-34] This compound has been known to possess a number of pharmacological activities such as hydrogen peroxide scavenging,[35] anti-atopic dermatitis,[36] anti-inflammation by IgG modulation and immune system protection.[37] Further, since over 55 years of EGF discovery, this first generation growth factor has been successfully progressed into clinical practice in the treatment of wounds.[38,39] Interestingly, the combination of hyaluronate and EGF can significantly improve the regeneration of skin tissues extending into the hypodermis.[40] In addition, estrogen-induced

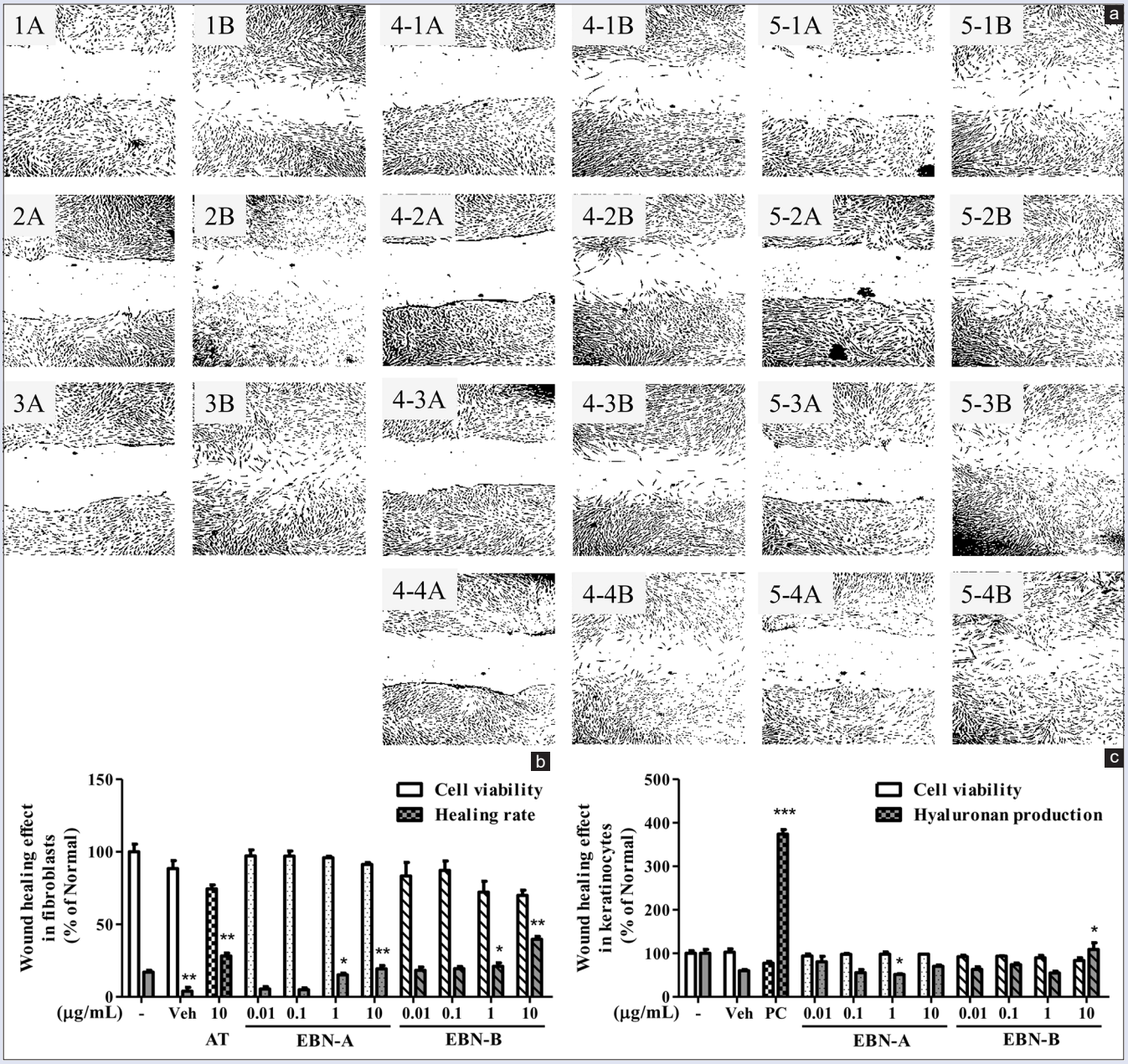

Figure 3: Wound-healing effect of edible bird's nests. (a) Fibroblast healing imaging; (b) Fibroblast healing rates; (c) Keratinocyte hyaluronan production. All data are shown as the mean ± standard deviation of at least three independent experiments performed in triplicate. Asterisks indicate highly significant differences from non-treated control. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$

EGF can induce pro-proliferative and anti-inflammatory effects during photoaging.[41] Moreover, EGF can protect the skin against atopic dermatitis, TNF-α/IFN-γ, or *Staphylococcus aureus*-induced inflammation.[42,43] Thus, EBN extracts enriched with sialic acid and EGF have potential applications in skin disease therapies such as wound healing that result in skin regeneration. Moreover, EBN-B containing higher contents of sialic acid and EGF was found to exert better biological effects such as anti-inflammatory and wound-healing activities in the present study.

In corneal keratocytes, Zainal Abidin *et al.* suggested that EBN can induce both cell proliferation and functional maintenance during corneal wound healing through the expression of aldehyde dehydrogenase, collagen type I and lumican.[6] Another study showed that EBN extract cream is useful for treating perineal wounds in *Rattus norvegicus*.[44] Similarly, the fibroblast migration speed with EBN-B at 10 μg/ml was greater than that with the positive control allatoin [Figure 3]. Hyaluronan is one of ECM components that plays a key role in tissue hydration and is beneficial for wound healing.[45] In this study, we found that the level of hyaluronan production by EBN-B was slightly better but was less than that with the positive control, retinoic acid [Figure 3]. Although, both allatoin and retinoic acid, which were used as positive controls, have little cytotoxic activity, EBN-A and EBN-B did not affect cell viability at levels up to 10 μg/ml in both HaCaTs and fibroblasts. In other words, EBN-B exerts a wound-healing effect by stimulating hyaluronan production. EBN can thus be considered as an alternative and safe material for enhancing wound healing.

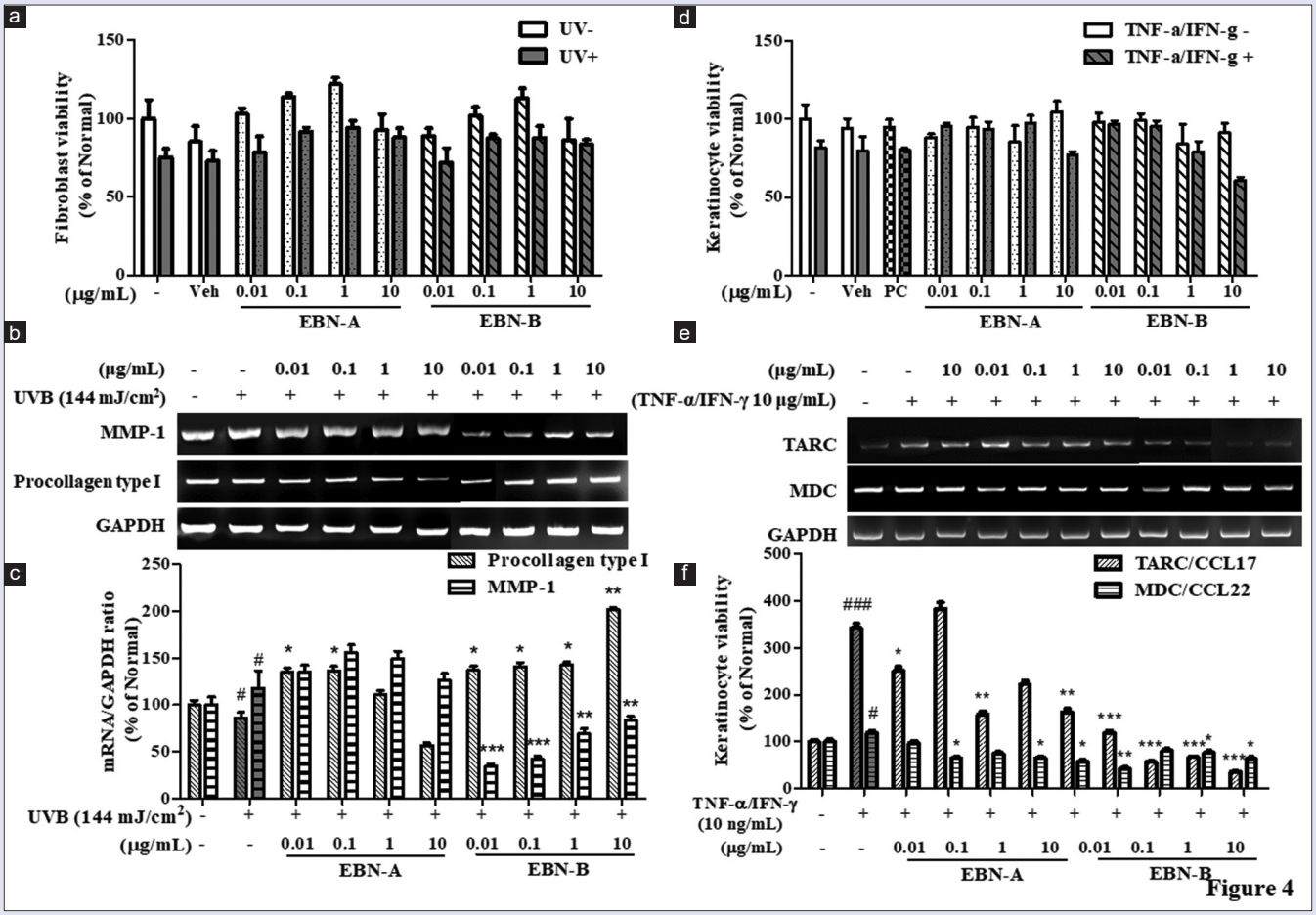

Figure 4: Anti-aging and anti-inflammatory activities of edible bird's nests against ultraviolet B or tumor necrosis factor alpha/interferon gamma. (a-c) Fibroblast cells were treated with ultraviolet B irradiation. (a) Cell viability; (b) The messenger RNA levels of matrix metalloproteinase-1 and type I procollagen with GAPDH as an internal control; (c) Analysis of messenger RNA expression. (d-f) Keratinocyte cells were treated with tumor necrosis factor alpha/interferon gamma. (d) Cell viability; (e) The messenger RNA levels of thymus-and activation-regulated chemokine and macrophage-derived chemokine with GAPDH as an internal control; (f) Analysis of messenger RNA expression. All data are shown as the mean ± standard deviation of at least three independent experiments performed in triplicate. # and * indicate significant differences, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, compared to the non-treated control; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared to the only ultraviolet B irradiated cells

In the photoaging process, UVB radiation upregulates MMP expression, which degrades collagen and other ECM proteins; UVB-induced MMP-1 overexpression initiates collagen breakdown by cleaving type I and type III collagen and plays an important role in the physiological mechanisms of skin photoaging. As expected, both EBN-A and EBN-B reduced MMP-1 and promoted collagen expression. As described above, these collagen regulating properties of EBN can contribute both anti-aging and wound-healing effects. Because Th2-type inflammation induces the release of MDC and TARC from macrophages and HaCaTs, MDC and TARC levels are correlated with the severity of atopic dermatitis.[46] High levels of these markers were recorded in the serum of atopic dermatitis patients.[47] However, EBN seemed to directly attack TARC and MDC leading almost diminishing their levels to those observed in healthy cells [Figure 4].

CONCLUSION

This study revealed that EBN possesses the ability to alleviate UVB-induced skin damage through regulation of MMP-1/procollagen type I expression. EBN blocked the overexpression of TARC/CCL-17 and MDC/CCL22 in TNF-α/IFN-γ-induced inflammatory HaCaTs. One of the most important effects investigated was that the wound-healing process could be significantly supported by treatment with EBN by promoting hyaluronan induction. EBN is thus a valuable product that represents a complementary and alternative medicine for skin disease treatment.

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Conflicts of interest

There are no conflicts of interest.

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