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Combination of Nicotinamide and *Agastache rugosa* Extract: A Potent Strategy for Protecting Hs68 Cells from UVB-Induced Photoaging

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ABSTRACT: This study investigated the protective effects of nicotinamide (NAM) and *Agastache rugosa* extract (AR) against ultraviolet B (UVB)-induced photoaging in Hs68 cells. The results demonstrated that NAM and AR, alone or in combination, exhibited concentration-dependent protective effects against UVB radiation. The highest synergistic effect was observed at a NAM:AR ratio of 6:4. This combination exhibited a synergistic protective effect against UVB-induced photoaging. The sample concentration required for 80% cell survival was 9.70 μM and 131.16 ppm for NAM and AR, respectively. However, when combined, they exhibited strong synergistic effects with concentrations as low as 0.11 μM and 17.50 ppm. Moreover, 5.26 μM of NAM and 1,082.13 ppm of AR were required to inhibit 30% of reactive oxygen species, but the combination treatment required 0.62 μM and 95.49 ppm, respectively. This combination significantly reduced the production of matrix metalloproteinase and increased collagen production. These findings highlight the potential of combining NAM and AR as functional cosmetic materials to protect against UVB-induced photoaging. The synergistic effects observed in this study provide valuable information for developing novel strategies for cosmetic combinations that target UVB-mediated skin damage.

Keywords: *Agastache rugosa*, aging, niacinamide, skin, synergy

INTRODUCTION

The skin primarily protects the body against external elements and is constantly exposed to ultraviolet radiation (Farage et al., 2008). Internal and external factors influence skin aging, which is typically categorized into two main types: intrinsic aging and photoaging (Tobin, 2017). Intrinsic aging primarily stems from genetic and endogenous factors that gradually manifest over time (Di Micco et al., 2021). It is often characterized by thin wrinkles, dryness, and loose and rough skin (Baumann, 2007). Meanwhile, photoaging is primarily caused by prolonged exposure to ultraviolet B (UVB) radiation and other environmental factors (Zhang et al., 2020). It is characterized by deep wrinkles, reduced skin elasticity, and pigmented spots. In skin photoaging, collagen degradation is a significant factor and primarily driven by the upregulation of matrix metalloproteinases (MMPs) within dermal fibroblasts, leading to compromised structural integrity of the extracellular matrix (Philips et al., 2011). Furthermore, chronic exposure to UVB irradiation induces reactive oxygen species (ROS) generation and increases skin cancer risk (Scharffetter-Kochanek et al., 2000). Considering the significance of ROS and MMPs in skin photoaging, natural materials that can inhibit ROS generation and downregulate MMPs have attracted considerable attention (Lee et al., 2019).

Agastache rugosa is a perennial plant belonging to the Lamiaceae family that is widely used for medicinal purposes (Park et al., 2020). It exerts various physiological activities, including anticancer, antidiabetes, anti-inflammation, antiaging, and antioxidation (Nan et al., 2022). *A. rugosa* contains several active compounds, including tilianin, luteolin, apigenin, diosgenin, rosmarinic acid, and acacetin (Lam et al., 2023). Among these compounds, rosmarinic acid (22 μg/g of dry weight) is the most abundant (Tuan et al., 2012). Because of the components of *A. rugosa*, many studies have explored their antioxidative properties and ability to reduce skin wrinkles and promote wound healing (Oh et al., 2016). Recently, *A. rugosa* extract (AR) has been used as a functional cosmetic ingredient to protect the skin from UV-induced damage

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(Shin et al., 2018).

Nicotinamide (NAM), the amide form of vitamin B3, is widely used in various dermatological applications because of its low toxicity, even at high doses (Damian, 2010). Various clinical trials have demonstrated that NAM-containing cosmetics effectively reduce UV-induced skin aging and pigmentation (Kawada et al., 2008; Fu et al., 2010). Moreover, topical NAM is reportedly beneficial in treating acne vulgaris because of its anti-inflammatory and healing activities (Rolfe, 2014). A previous study showed that NAM can delay the accumulation of harmful ROS and other signs of aging, including telomere shortening and senescence-associated β-gal activity (Kang et al., 2006).

In the field of phytomedicine, scientific research has increasingly focused on synergistic studies, which have led to the identification of novel key activities (Wagner and Ulrich-Merzenich, 2009). A synergistic effect refers to the phenomenon wherein the combined action of two or more agents results in a more significant impact than the sum of their individual effects (Chou, 2018). Synergistic combinations have been recognized for their ability to enhance efficacy, reduce toxicity, and provide a multitarget approach (Zhou et al., 2016). Several studies have explored the potential of natural product combinations to improve inflammation and oxidative stress. However, the specific synergistic effect of NAM and AR combination in addressing photoaging remains unclear (Zhou et al., 2022). Most skincare products contain NAM. Therefore, discovering ingredients or extracts that induce synergistic protective effects could significantly contribute to the development of functional cosmetic compositions. The present study investigated the anti-photoaging effect of NAM-AR combinations on UVB-induced skin fibroblasts to gain insights into their synergistic effect and potential application in preventing skin aging.

MATERIALS AND METHODS

Materials

NAM, 2',7'-dichlorofluorescein diacetate (DCFH-DA), dimethyl sulfoxide, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich. Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), ethylenediaminetetraacetic acid, and penicillin-streptomycin were obtained from HyClone Laboratories, Inc..

Sample combination

A. rugosa was extracted with water for 24 h in a shaker (Eyela Model MMS-300, Tokyo Rikakikai), filtered through Toyo No. 2 filter paper (Toyo Ltd.), and stored at −20°C until use. The extraction yield was 26.2%. NAM and AR (NAM-AR) mixtures were prepared at different volume ratios (9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, and 1:9 v/v). The ratios were determined on the basis of a previous study (Kim et al., 2023). Subsequently, the mixtures were diluted in media and subjected to serial dilutions.

Cell culture and sample treatment

Hs68 cells were cultured in DMEM containing 10% heat-inactivated FBS, 100 units/mL of penicillin, and 50 μg/mL of streptomycin and maintained at 37°C in 5% CO_2. Human skin fibroblasts were seeded in 96-well plates at a density of 5.0×10^4 cells/mL. Then, they were pretreated with test samples in serum-free medium for 24 h. The cells were rinsed with phosphate buffered saline and exposed to UVB irradiation (30 mJ/cm^2) using a UVB lamp (GL20SE, Sankyo Denki Lamps). The radiation intensities were monitored using a UV light meter (UV-340A, Lutron, Taiwan). After irradiation, the cells were maintained in serum-free medium for an additional 24 h with or without samples. Next, MTT assay was performed to assess cell viability and protective effects. The cells were incubated with MTT reagent (0.5 mg/mL) for 2 h and a spectrophotometer (BioTek Instruments Inc.) was used to measure the absorbance at 550 nm.

Measurement of ROS, MMP-1, MMP-3, and total collagen levels

Intracellular ROS levels were quantified using a DCFH-DA fluorescent probe in accordance with a previously described method (Lee et al., 2019). After UVB irradiation, the cells were treated with or without samples in serum-free media for an additional 30 min and stained with 25 μM DCFH-DA. A fluorescence spectrophotometer (LS-55, PerkinElmer) was used to measure the fluorescence intensity corresponding to intracellular ROS generation for 2 h at an excitation wavelength and emission wavelength of 485 and 530 nm, respectively. MMP-1 and MMP-3 levels were determined using enzyme-linked immunosorbent assay kits (Merck & Co., Inc.). Collagen production was measured using the Sircol Soluble Collagen Assay Kit (Biocolor).

Synergism experiments

The synergistic effects were evaluated using the previously described combination index (CI) model (Chou, 2018). The interaction of combinations was calculated using "CompuSyn" software (CompuSyn, Inc.).

Statistical analysis

Statistical analysis was performed using one-way analysis of variance followed by Duncan's test using SAS version 9.4 (SAS Institute Inc.). The results are expressed as mean±standard error, and the experiments were conducted independently at least three times.

RESULTS AND DISCUSSION

Protective activities of NAM and AR against UVB-induced photoaging in Hs68 cells

We assessed the protective effects of NAM, AR, and NAM-AR mixtures against UVB-exposed skin damage in Hs68 cells to investigate their synergistic activities in inhibiting skin photoaging. The cell viability did not significantly change with the treatment of NAM and AR at concentrations up to 5 μM and 100 ppm, respectively, or with their combination (Fig. 1A and 1D). Exposure to UVB radiation (30 mJ/cm^2) reduced cell viability compared with that of control cells (Fig. 1B). However, NAM (5 μM) or AR treatment (100 ppm) restored cell viability by 27.5% and 26.2%, respectively, compared with UVB-irradiated cells. UVB-exposed human skin fibroblasts were treated with NAM-AR mixtures (10:0, 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9, and 0:10 v/v) to confirm the optimal combination ratio for protective effects (Fig. 1C). The NAM-AR combination ratio of 6:4 showed the highest protective activity against UVB irradiation compared with other ratios. After observing the positive impact of the NAM-AR combination ratio of 6:4, we observed an increase in cell viability, even when the concentration was diluted by a factor of eight (Fig. 1E). Consequently, we used a NAM-AR combination ratio of 6:4 for subsequent

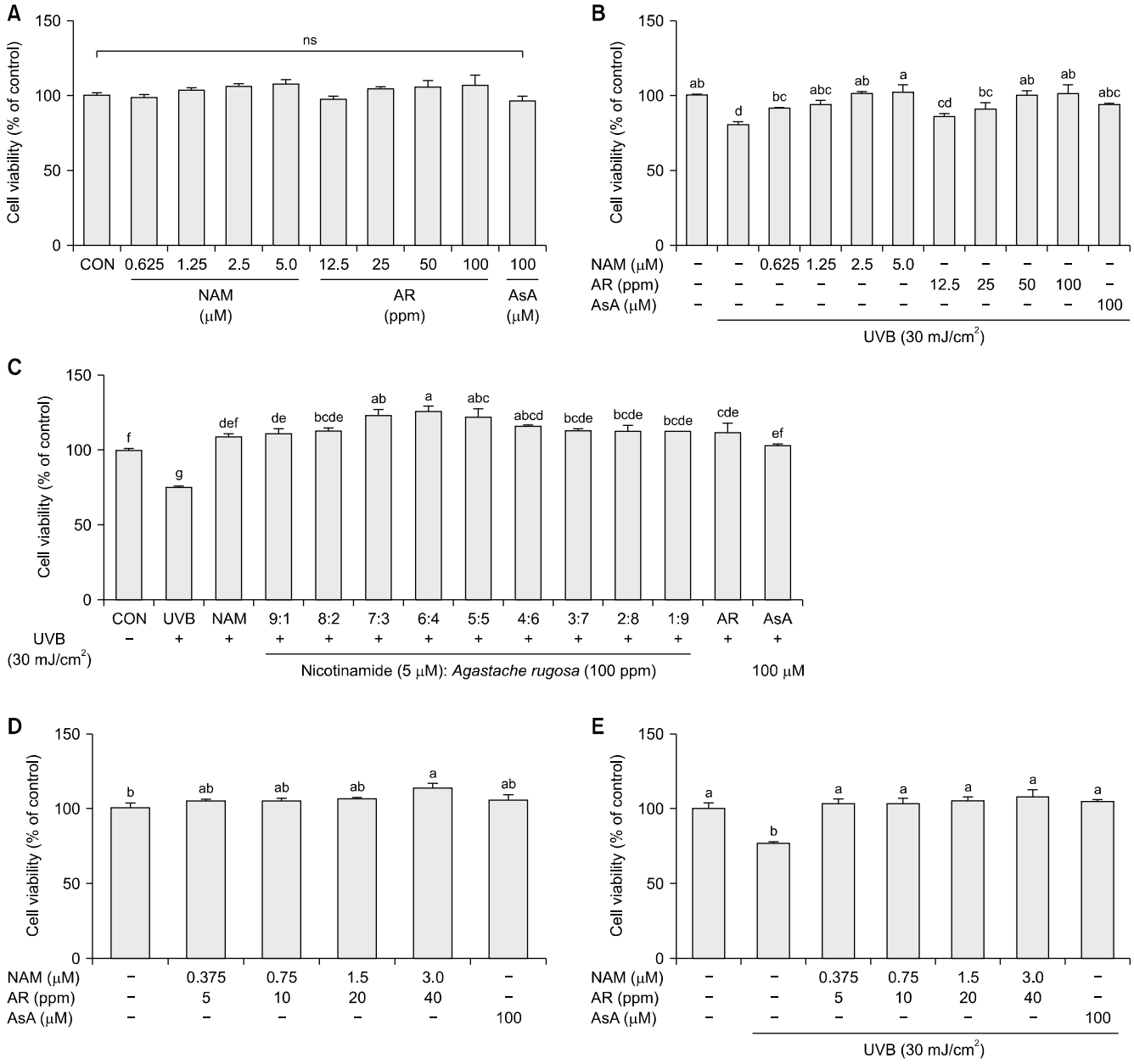

Fig. 1. Cell viability (A and D) and protective effects (B, C, and E) of nicotinamide (NAM), *Agastache rugosa* extract (AR), and NAM and AR (NAM-AR) mixture in Hs68 cells. Ascorbic acid (AsA) (100 μM) was used as a positive control. Each value is expressed as the mean±SE (n=3). Different letters above the bars (a-g) indicate significant differences on the basis of Duncan's test (P<0.05). ns, not significant; UVB, ultraviolet B.

experiments. As shown in Fig. 2, the UVB-irradiated group exhibited a significant increase in ROS generation compared with the control group. However, treatment with the NAM-AR mixture with a combination ratio of 6:4 decreased ROS production compared with treatment with NAM (5 μM) or AR alone (100 ppm). Our findings suggested that the NAM-AR mixture with 6:4 combination ratio is more effective in preventing increased ROS production than treatment with either compound alone.

Previous studies have emphasized the potential of natural bioactive compounds to address skin aging concerns. Moreover, researchers have explored strategies to create safe and effective cosmetic formulations for skin protection. Among the various approaches, the combination of natural sources has gained significant interest because of their potential synergistic effects and enhanced therapeutic benefits (Zhou et al., 2016). Phenolic compounds are associated with the therapeutic effects of various medicinal plants. The major phenolics of AR, including tilianin, acacetin, apigenin, quercetin, rosmarinic acid, caffeic acid, and chlorogenic acid, have been previously reported (Nechita et al., 2023). Several phytochemicals, antioxidants, and AR extracts have been reported to possess anti-inflammatory, antioxidative, and collagen-boosting properties that are essential for maintaining skin health and combating the signs of aging (Oh et al., 2016). Furthermore, AR extract has demonstrated radical-scavenging activity against free radicals and superoxide, reducing intracellular ROS levels (Lee et al., 2020). NAM is known for its antiaging properties, including the ability to improve skin elasticity, reduce wrinkles, and enhance skin tone (Boo, 2021). According to a previous study, NAM prevents UV-induced skin damage by enhancing DNA repair and reducing immune system inhibition (Snaidr et al., 2019). Moreover, NAM exerts photoprotective effects against immunosuppression and carcinogenesis in mice and humans when used as a lotion or administered orally (Damian, 2010). UV radiation exposure depletes cellular energy, and NAM can replenish this energy in irradiated skin cells. Altogether, the NAM-AR mixture with 6:4 combination ratio can improved UVB-induced damage by modulating ROS levels or the immune system, which affects the maintenance of the normal redox status of human skin fibroblasts.

Synergistic effect of NAM-AR mixture

As defined in a previous study, synergy refers to the remarkable phenomenon that arises when two or more drugs or compounds are combined, resulting in a collective effect that surpasses the sum of their individual effects (Chou, 2010). By contrast, an additive effect is characterized by the summation of individual drug effects with no interaction (Roell et al., 2017). It is important not to confuse synergistic effects with additive effects. A more intricate mathematical algorithm must be applied to distinguish these effects accurately. The present study evaluated the interaction between NAM and AR using CompuSyn software. The amounts of NAM, AR, and NAM-AR required to reach 80% cell viability were 9.70 μM, 131.16 ppm, and 0.11 μM with 17.50 ppm, respectively. A dose-dependent response was observed when assessing the efficacy of NAM and AR, as higher concentrations of these compounds led to an increased protective effect on skin fibroblast viability (Fig. 3A and 3B). The NAM-AR combination exhibited a more protective effect than NAM or AR alone, as determined by median effect analysis (Fig. 3C). According to the fraction affected-CI curve, the combination of the two substances showed a synergistic effect when CI values were less than 1. As shown in Fig. 3D, the NAM-AR mixture with 6:4 combination ratio exhibited CI values less than 1 (ranging from 0.15 to 0.21) in protective effect. CI values less than 1 were observed for all four pairs of combinations, indicating that the NAM-AR combination exhibited syn-

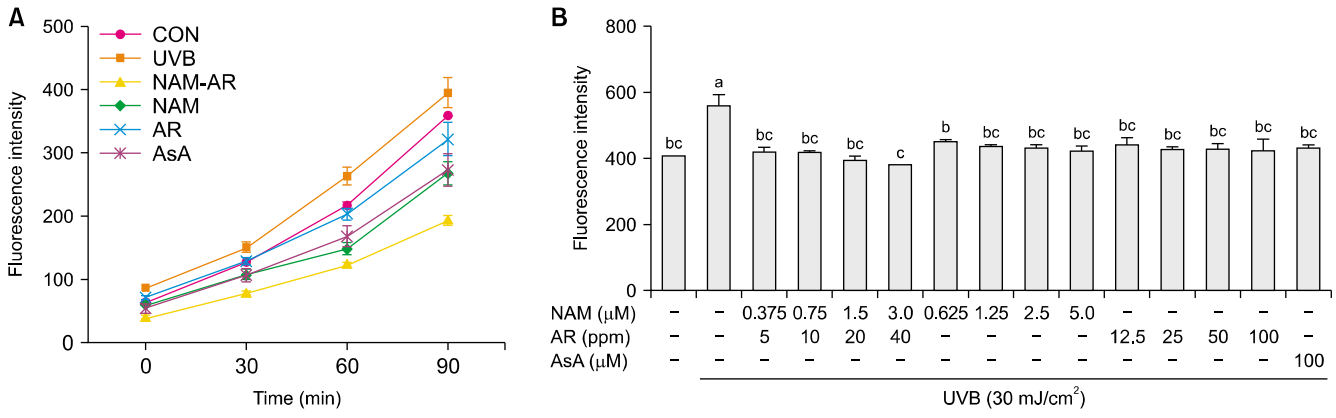

Fig. 2. Effects of nicotinamide (NAM), *Agastache rugosa* extract (AR), and NAM and AR (NAM-AR) mixture with 6:4 combination ratio on the time course of reactive oxygen species (ROS) generation (A) and ROS production (B) at 120 min in ultraviolet B (UVB)-induced Hs 68 cells. Ascorbic acid (AsA) (100 μM) was used as a positive control. Each value is expressed as the mean±SE (n=3). Different letters above the bars (a-c) indicate significant differences on the basis of Duncan's test (P<0.05).

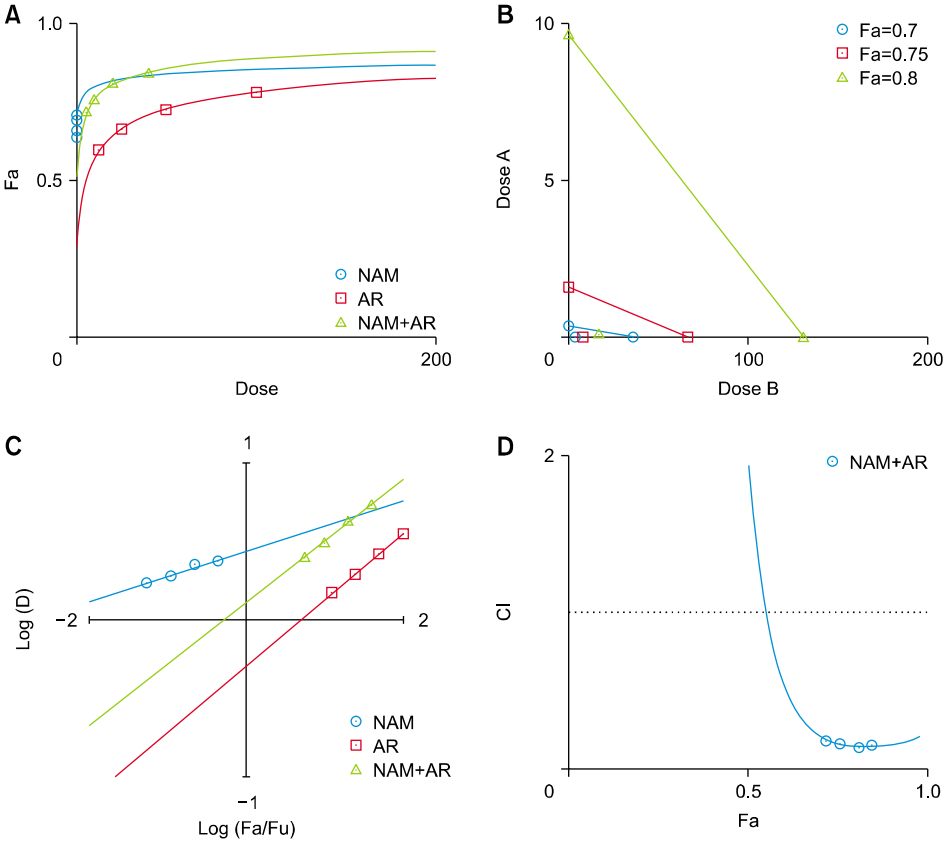

Fig. 3. (A) Dose-effect curves of nicotinamide (NAM), *Agastache rugosa* extract (AR), and NAM and AR (NAM-AR) mixture with 6:4 combination ratio on skin fibroblast viability. (B) Isobologram curves of NAM, AR, and NAM-AR mixture with 6:4 combination ratio. (C) Median effect plot of NAM, AR, and NAM-AR mixture with 6:4 combination ratio. (D) Combination index (CI) plot.

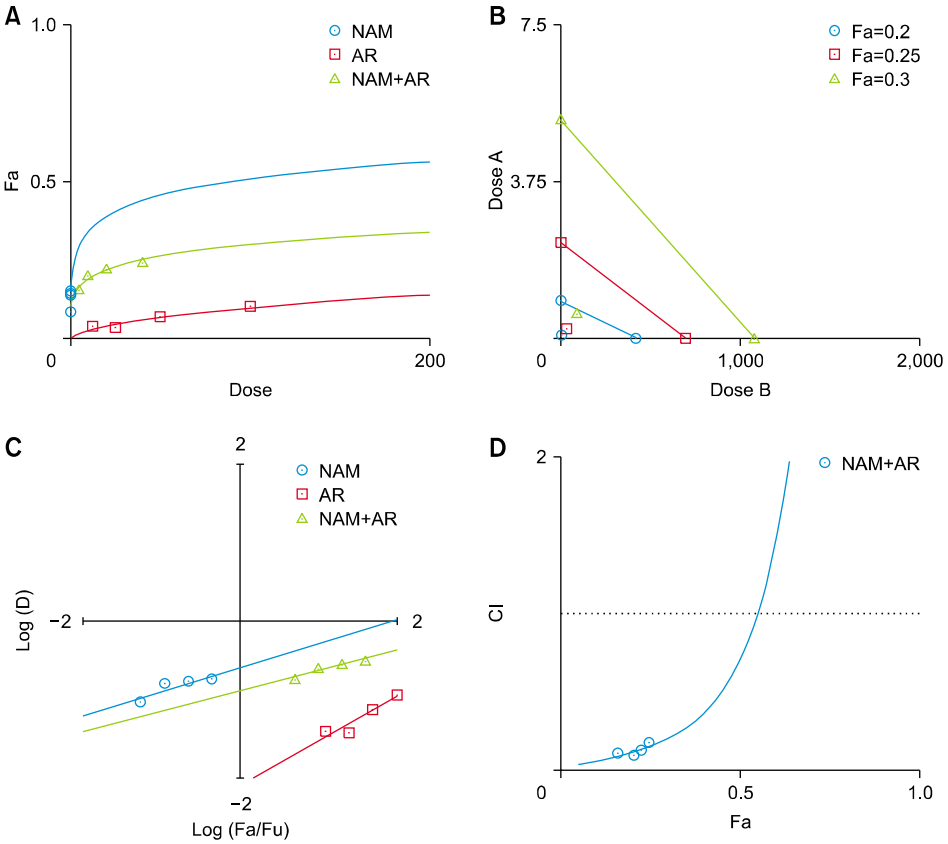

Fig. 4. (A) Dose-effect curves of nicotinamide (NAM), *Agastache rugosa* extract (AR), and NAM and AR (NAM-AR) mixture with 6:4 combination ratio on reactive oxygen species production. (B) Isobologram curves of NAM, AR, and NAM-AR mixture with 6:4 combination ratio. (C) Median effect plot of NAM, AR, and NAM-AR mixture with 6:4 combination ratio. (D) Combination index (CI) plot.

ergism in Hs68 cells.

The NAM-AR combination required lower amounts (0.62 μM and 95.49 ppm, respectively) required to reach 30% ROS inhibition than concentrations needed for single treatment of NAM (5.26 μM) or AR (1,082.13 ppm) (Fig. 4A and 4B). The inhibitory effect of NAM-AR on

ROS generation was compared with that of NAM or AR alone (Fig. 4C). The NAM-AR mixture with 6:4 combination ratio exhibited a CI less than 1 (0.12～0.21) in ROS inhibition (Fig. 4D). Our findings demonstrated that synergy occurs during treatment with NAM-AR mixture with 6:4 combination ratio and that applying this combination to prevent skin photoaging may offer more benefits than using a single compound or extract alone.

Effect of NAM-AR mixture on MMPs and total collagen production

MMPs, which are a group of widely distributed endopeptidases, can break down extracellular matrix proteins (Verma and Hansch, 2007). Among them, MMP-1 is the primary enzyme responsible for initiating collagen fiber breakdown in human skin. Following the cleavage of MMP-1, MMP-3 and MMP-9 further degrade collagen (Brennan et al., 2003). A previous study found that MMP-1, MMP-3, and MMP-9 levels are elevated in aged human skin (Quan et al., 2013). Furthermore, ROS play a crucial role in increasing MMP levels in the skin (Quan and Fisher, 2015). This leads to progressive collagen breakdown in the dermis, which contributes to skin aging. Therefore, decreasing MMP levels may effectively alleviate skin wrinkling and sagging. As shown in Fig. 5, UVB irradiation significantly increased MMP-1 and MMP-3 levels and decreased total collagen levels compared with those in the control group. However, NAM and AR treatment markedly suppressed MMP-1 and MMP-3 production and enhanced total collagen production. Moreover, the NAM-AR mixture with 6:4 combination ratio synergistically reduced the UVB-induced increase in MMP-3 levels and decreased collagen levels compared with NAM or AR treatment alone. According to a previous study, NAM possesses antioxidant properties, can reduce pigmentation and wrinkles, enhance the growth of dermal and epidermal cells, and contribute to collagen production (Chen and Damian, 2014). Yun et al. (2019) reported that AR attenuates UVB-induced aging by modulating the transforming growth factor-β/Smad and mitogen-activated protein kinase/activator protein 1 pathways in hairless mice. In another study, AR treatment reduced ROS overproduction by promoting antioxidant enzyme expression (Seo et al., 2019).

Therefore, a multifaceted approach involving various cellular mechanisms is required to prevent skin aging. Stimulating collagen production is one crucial aspect (Seo et al., 2019). Additionally, regulating oxidative stress and free radicals reduces skin damage and mitigates wrinkling, ultimately contributing to overall skin health (Oh et al., 2016). Another mechanism involves the inhibition of chronic inflammation, which significantly reduces the adverse effects of inflammation-related skin damage (Seo et al., 2019). Simultaneously, strengthening the skin's protective barrier helps shield it from external factors, effectively preventing skin damage and contributing to antiaging efforts (Lee et al., 2020). Repairing DNA damage within skin cells is essential for sustaining proper cellular function and ultimately preventing premature skin aging (Dong et al., 2008). The NAM-AR mixture with 6:4 combination ratio may improve skin photoaging through various cellular mechanisms involving NAM and AR. However, further studies are required to confirm these underlying mechanisms using *in vitro* and *in vivo* models.

In conclusion, our research demonstrates that the NAM-AR mixture with 6:4 combination ratio exhibits synergy by enhancing the protective effect and reducing ROS generation in UVB-exposed Hs68 fibroblasts. This combined treatment alleviated the increase in MMPs and decrease in collagen compared with individual treatment. These results underscore the possibility of developing innovative skincare therapies that harness the power of NAM and AR in a unique blend, offering a more effective shield against UVB-induced damage and aging. This study opens new avenues for further exploration and application in the fields of skincare and antiaging treatments.

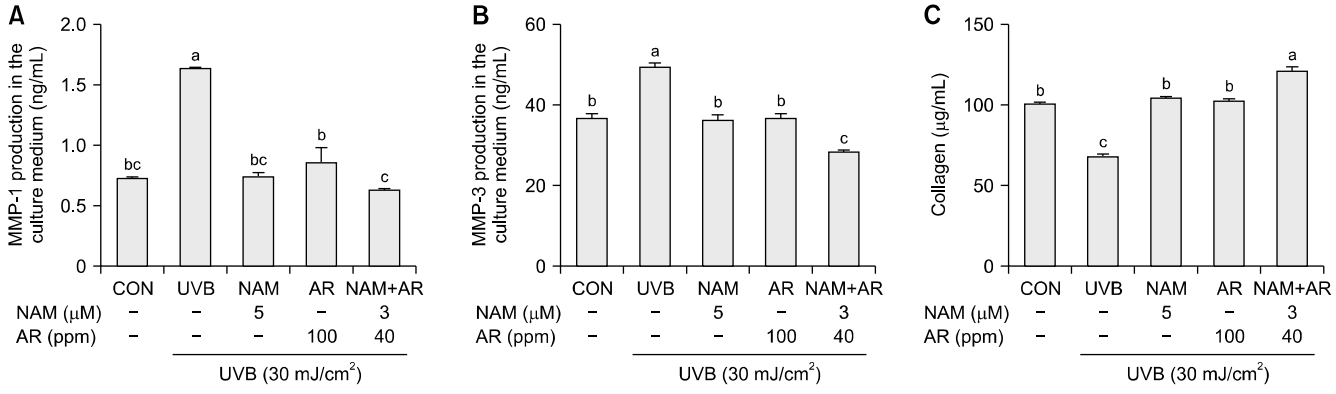

Fig. 5. Effect of nicotinamide (NAM), *Agastache rugosa* extract (AR), and NAM and AR (NAM-AR) mixture with 6:4 combination ratio on matrix metalloproteinase (MMP)-1 (A), MMP-3 (B), and total collagen production (C) in ultraviolet B (UVB)-induced Hs68 cells. Each value is expressed as the mean±SE (n=3). Different letters above the bars (a-c) indicate significant differences on the basis of Duncan's test (P<0.05).

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None.

AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Concept and design: SB, HL. Analysis and interpretation: SB. Data collection: SB, HH, SH. Writing the article: SB, HL. Critical revision of the article: HL, HSJ, JL. Final approval of the article: all authors. Statistical analysis: SB, HL. Overall responsibility: SB, HL.

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