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ORIGINAL CONTRIBUTION

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Clinical evaluation of the safety and efficacy of a timosaponin A-III-based antiwrinkle agent against skin aging

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Abstract

Background: Timosaponin A-III (TA-III) is known to exist in the medicinal herb of *Anemarrhena asphodeloides* as one of major chemical components.

Aims: The photoprotective properties of TA-III on UVB-exposed HaCaT cells were evaluated on the antiwrinkle effects and skin safety in terms of clinical trial.

Methods: The level of matrix metalloproteinase (MMP)-1, tissue inhibitor of metalloproteinases (TIMPs), and pro-inflammatory cytokines were measured in HaCaT cells following UVB irradiation. To evaluate the clinical safety of an agent containing 0.25% of TA-III for use on human skin. Female subjects (n = 21) between the ages of 43 and 55 who met the criteria for subject selection were selected. They were beginning to form or had already formed wrinkles.

Results: UVB irradiation increased MMP-1 expression and pro-inflammatory cytokines. These increases were attenuated by TA-III pretreatment of UVB-exposed HaCaT cells. We found that the agent containing 0.25% of TA-III ameliorated skin wrinkling. A comparison between groups showed that wrinkle parameters were significantly reduced after 12 weeks of product use ($P < 0.05$). According to skin safety result, TA-III showed no dermatological toxicity was found in participants.

Conclusions: In conclusion, TA-III could provide protection against photoaging and daily application of TA-III for 12 weeks significantly reduced signs of facial aging by limiting wrinkle formation.

KEYWORDS

Anemarrhena asphodeloides, clinical trial, skin wrinkle, timosaponin A-III

1 | INTRODUCTION

There are two categories of aging process with intrinsic and extrinsic aging. The former is represented by internal factors as accompanied by genetic events, and the latter is related to external factors such as stress, radiation, pollution, and smoking.[1] Aging skin has features in changes in skin thickness, epidermal and dermal layer which appear as wrinkles, and structural abnormalities including degeneration of dermal elastic fibers.[2] Clinically, the skin becomes coarse, then thins, and becomes lax, showing sallowness, wrinkles, irregular hyperpigmentation, lentigines, and telangiectasias.[3]

As people grow older, they become interested in the condition of their skin.[4] Skin wrinkles are typical phenomena of aging with morphological changes in relation to dermal function with breakdown

and generation of collagen.[5] Wrinkle researches have been developed with animal and in vitro models.[6] Keratinocytes release inflammatory cytokines including interleukin (IL)-1, IL-6, IL-8, IL-10, and tumor necrosis factor (TNF)-α upon exposure to UV.[7] UVB-induced cell damage leads to activation of reactive oxygen species (ROS)-sensitive signaling pathways, such as the mitogen-activated protein kinase (MAPK) pathway.[8] This pathway is an important mediator of inflammatory cytokine induction in response to UVB irradiation.[9]

Various cosmetics can inhibit skin wrinkles, distinct from drugs, which are used for a set period for the treatment of medical conditions.[10] It is important to secure the safety of cosmetics because they are routinely used for a long time.[11] The safety of skin applications is determined individually based on the ingredients used.[12] It is performed on the subjects who have normal skin, and the appearance of erythema, edema, swelling, and papules is evaluated by experts to determine product safety.[13]

The rhizomes of *Anemarrhena asphodeloides* Bunge (Liliaceae) have been used in traditional medicine as an antidiabetic, an antipyretic, and an antidepressant in China, Japan, and Korea. It is also used to treat febrile disease in clinical practice in China.[14,15] Various chemical constituents are reported to be present in *A asphodeloides*, among which timosaponin A-III (TA-III) has various pharmacological efficacy and is used as an antidiabetic, antipyretic, and antidepressive material in traditional medicine.[15-18] In previous research, TA-III inhibits metastasis.[19] However, there is no study to protect photoaging caused by UVB.

In order to identify potential antiwrinkle agent, various compounds from *A asphodeloides* were evaluated in HaCaT cells. In skin clinical trials, the safety of TA-III and the effect of inhibiting skin wrinkles were examined and confirmed for use as cosmetics for skin wrinkle prevention.

2 | MATERIALS AND METHODS

2.1 | Isolation process

Timosaponin A-III (TA-III; Figure 1) was isolated as previously described.[20]

2.2 | UVB irradiation and cell viability assay

An immortalized nontumorigenic human keratinocyte cell line (HaCaT) (1×10^4) was seeded into 96-well culture plates and exposed to 20 mJ/cm^2 of UVB in the presence or absence of TA-III for 24 hr Immediately after UVB irradiation, cell viability was assessed by incubation with 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) for 1 hr and measuring its reduction to formazan, according to the manufacturer's instructions; samples were assayed at 490 nm using a microplate spectrophotometer (Molecular Devices).

2.3 | Analysis of MMP-1 secretions by ELISA

After UVB irradiation, MMP-1 levels in the culture media of HaCaT cells (5×10^4) were determined using human total MMP-1 ELISA kits according to the manufacturer's instructions. HaCaT cells were seeded and treated with extracts and various compounds (timosaponin A-III, timosaponin B, timosaponin B-II, anemarsaponin B, anemarsaponin E, and timosaponin C) from *A asphodeloides*. To perform quantification of MMP-1 secretions induced by UVB exposure, the culture supernatant was measured by colorimetric analysis.

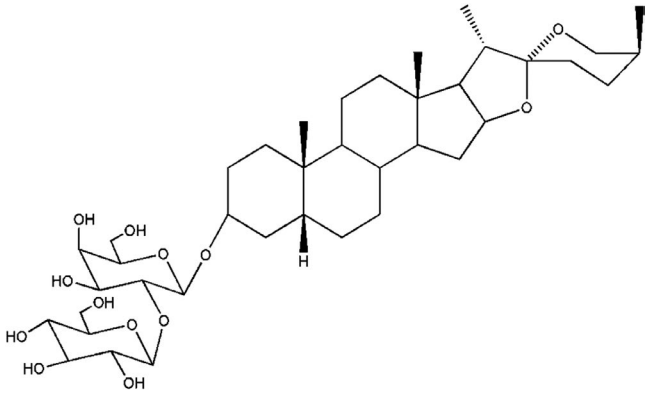


FIGURE 1 Chemical structure of timosaponin A-III, isolated from *Anemarrhena asphodeloides*

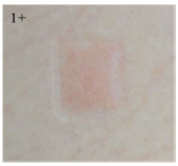
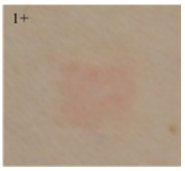
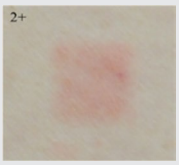
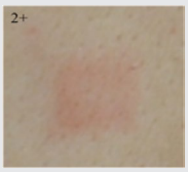
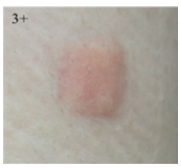
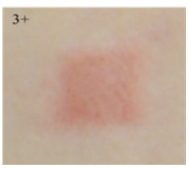
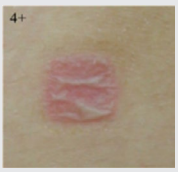
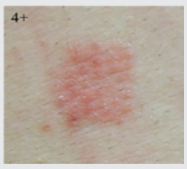
2.4 | Quantitative real-time polymerase chain reaction (qRT-PCR) assays

Total RNA was extracted from each sample using TRIzol (Invitrogen). qRT-PCR was performed using TaqMan assays (Applied Biosystems) specific for TIMP, IL-1β, IL-8, and TNF-α (TIMP1; Hs00171558_m1, IL-1β; Hs00174097_m1, IL-8; Hs00174103_m1, TNF-α; Hs01113624_g1) on a QuantStudio™ 6 Flex Real-Time PCR system. Each sample was assayed in triplicate, and relative mRNA expression levels were calculated using the ΔΔCt method and normalized to the β-actin mRNA level in each sample.

TABLE 1 Percent composition (w/w) of the formulations applied to skin

Components	Vehicle group (%)	TA-III group (%)
Water	84.80	84.55
Dipropylene glycol	5.00	5.00
Disodium EDTA	0.02	0.02
Phenoxyethanol	0.30	0.30
Carbomer	0.20	0.20
Glyceryl stearate	1.50	1.50
Sorbitan stearate	0.50	0.50
Polysorbate 60	1.50	1.50
Cetearyl alcohol	1.50	1.50
Cetyl ethylhexanoate	2.50	2.50
Sodium hydroxide	0.080	0.08
Water	2.00	2.00
Fragrant	0.10	0.10
TA-III	—	0.25
Distilled water	100	100

TABLE 2 Clinical standard photographs of visual assessment for human patch test

Description	After 30 min	After 24 h
Slight erythema, either spotty or diffuse		
Moderate uniform erythema		
Intense erythema with edema		
Intense erythema with edema and vesicle		

2.5 | Clinical safety test

2.5.1 | Human subjects and methods

A total of 30 subjects were recruited and conducted in accordance with the guideline of Ministry of Food and Drug Safety in Korea based on Declaration of Helsinki, as appropriate. Timosaponin A-III (TA-III) was prepared as described previously.[20] An agent containing 0.25% of TA-III (Table 1) was applied as a test solution (test group). The patch was left on the testing area for 48 h containing 20 μL of the test solution, and upon removal of the patch, the testing area was marked with a skin marker. Each testing area was observed after 30 min and 24 hr The patch test has been done including positive (0.5% SLS, sodium lauryl sulfate) and negative (Squalane) controls.

2.5.2 | Limitations and assessment criteria

While the patches were in place, the subjects were instructed not to let the testing area (back) come into contact with water. Observations were made 30 min and 24 hr after patch removal, and skin reactions were assessed according to the criteria of the Frosch & Kligman method[21] and the guidelines of the Personal Care Product Council (PCPC; Table 2).[22]

2.5.3 | Calculation of results

The average reactivity at 48 and 72 hr was calculated using the equation below, and the average reactivity to each individual solution was determined according to the Frosch & Kligman criteria (Table 3).

TABLE 3 Human primary irritation index for cosmetic products[12]

Range of response	Criteria
$0.00 \le R < 0.87$	Slight
$0.87 \le R < 2.42$	Mild
$2.42 \le R < 3.44$	Moderate
$3.44 \le R$	Severe

TABLE 4 Visual assessment using a modified version of Danielle's criteria

Grade*	Description criteria
0	There are no skin wrinkles, and skin texture is delicate
1	Thin wrinkles on the skin are starting to become visible
2	Thin wrinkles on the skin are visible
3	There are many thin wrinkles on the skin, and moderate wrinkles are starting to become visible
4	Moderate wrinkles on the skin are visible
5	There are moderate wrinkles, but deep wrinkles are not visible
6	There are many moderate wrinkles on the skin, and deep wrinkles are starting to become visible
7	Deep wrinkles on the skin are visible
8	There are many deep wrinkles on the skin
9	There are excessively numerous deep wrinkles on the skin

*Wrinkles were assessed in 0.5-unit increments.

$$\text{Response} = \frac{\Sigma\,(\text{Grade} \times \text{No. of Responders})}{4\ (\text{Maximum grade}) \times n\ (\text{Total Subjects})} \times 100 \times 1/2$$

2.6 | Antiwrinkle clinical trials

2.6.1 | Patients and methods

The study was conducted in accordance with the intent and purpose of good clinical practice regulations as described in Korean Good Clinical Practice (KGCP) and/or the Declaration of Helsinki, as appropriate. The study protocol was approved by DERMAPRO Ltd., Institutional Review Board (1-220777-A-N-02, Protocol approval No.: 1-220777-A-N-02-DICN15088). Furthermore, this study protocol was followed functional cosmetic guideline of Ministry of Food and Drug Safety in Korea. This study has been uploaded to http://cris.nih.go.kr/cris/index.jsp (identifier KCT0002223) from National Research Institute of Health. The subjects of this study were women aged 30-65 years, who met the inclusion criteria. Subjects who had started to develop wrinkles or who already had wrinkles, as defined by functional cosmetic guideline criteria, were explained the purpose and procedure of this study. Written consent was obtained from subjects who were interested in this study in advance and was informed of expected efficacy and potential adverse events.

For this study, 21 women between the ages of 43 and 55 were selected. Subjects who were beginning to form or had already formed wrinkles, according to the SOP of the DermaPro Institute of Dermatological Sciences, were targeted. Those with the intention to participate were given study-participation consent forms to fill out and were enrolled in the study. They were healthy without acute and chronic diseases including skin diseases, who voluntarily signed the written consent after hearing an explanation of the purpose and details of the study, and who would be able for follow-up assessments during the testing period.

The subjects were informed that no other functional cosmetics or drugs should be used with the test product. Subjects were told that they could use basic cosmetic products in addition to the test

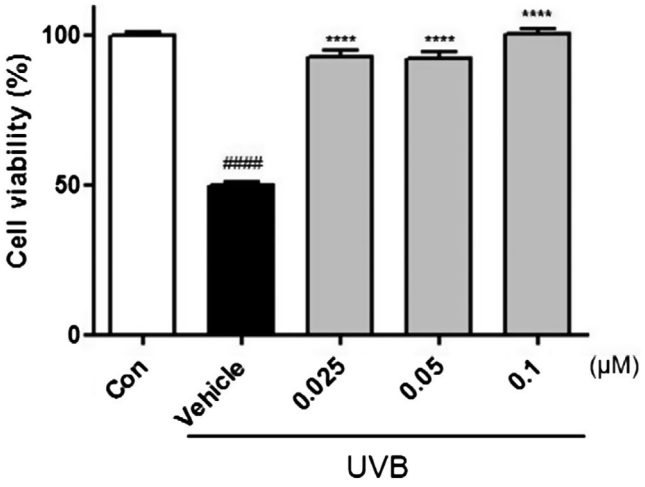
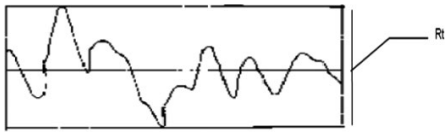
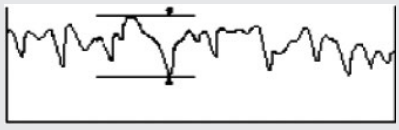
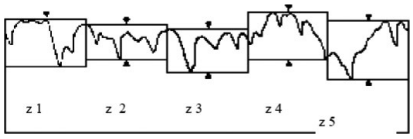
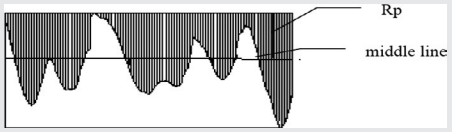
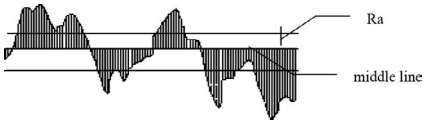


FIGURE 2 Effects of TA-III on cell viability in HaCaT cells after UVB irradiation. HaCaT cells were pretreated with TA-III at concentrations indicated for 24 h, followed by UVB irradiation. Data are reported as percentages. $^{\#\#\#\#}P < 0.0001$ for the comparison with the control group and $^{****}P < 0.0001$ for the comparison with the vehicle group

TABLE 5 Diagram and definition of wrinkle parameters

Parameters	Description criteria
	Skin roughness Rt
	Maximum roughness Rm
	Average roughness Rz
	Smoothness depth Rp
	Arithmetic average roughness Ra

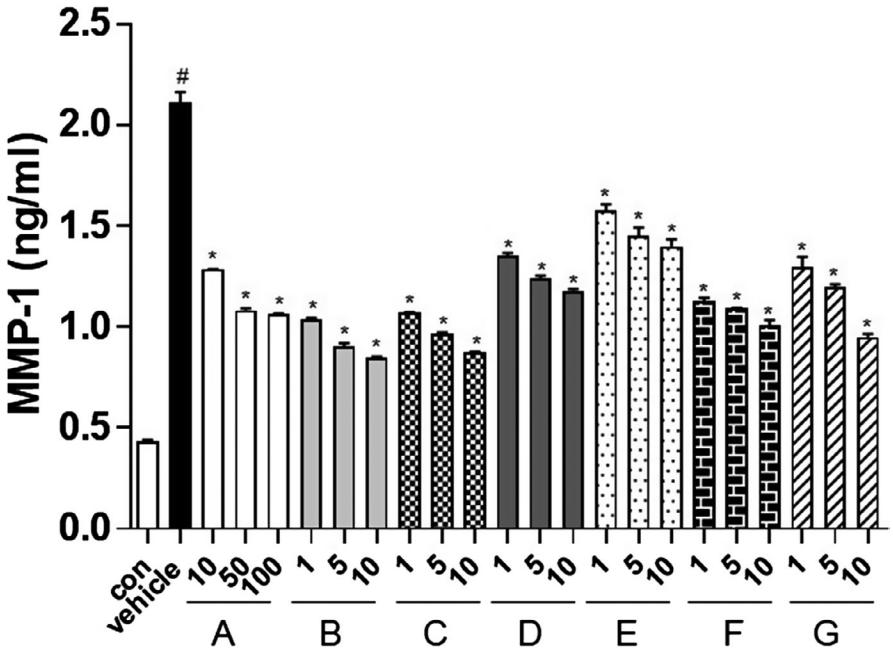


FIGURE 3 TA-III attenuated UVB-induced effects on MMP-1 expression in HaCaT cells. A: *Anemarrhena asphodeloides* extracts and various compounds (B: timosaponin A-III, C: timosaponin B, D: timosaponin B-II, E: anemarsaponin B, F: anemarsaponin E, and G: timosaponin C) treated with HaCaT cells for 24 h before UVB irradiation. Concentration of *A asphodeloides* extracts was μg/mL, and other compounds of concentrations were μmol/L. $^{\#}P < 0.05$ for the comparison with the control group and $^{*}P < 0.05$ for the comparison with the vehicle group

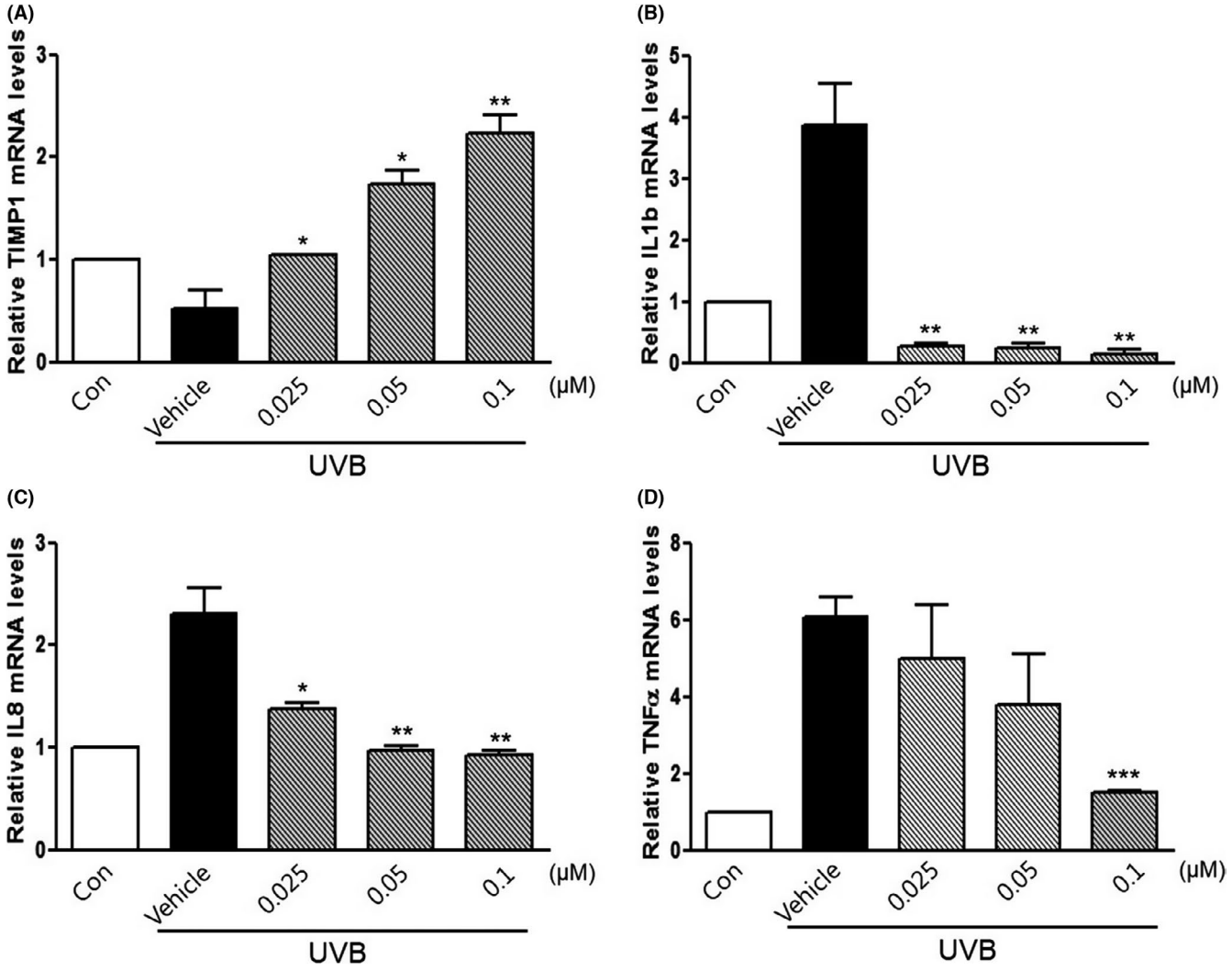


FIGURE 4 TA-III treatment attenuated UVB-induced effects on TIMP and pro-inflammatory cytokine expression levels in HaCaT cells. (A) TIMP, (B) IL-1β, (C) IL-8, and (D) TNF-α, mRNA levels were determined by qRT-PCR analysis. $^{***}P < 0.001$, $^{**}P < 0.01$, and $^{*}P < 0.05$ for the comparison with the vehicle group

TABLE 6 Skin characteristics of subjects (n = 30)

Items	Classification	Frequency (n)	Percentage (%)
Skin type	Dry skin	13	43.33
	Normal skin	11	36.67
	Oily skin	0	0.00
	Dry to oily skin	6	20.00
	Problem skin	0	0.00
Irritability	Yes	3	10.00
	No	27	90.00
Stinging	Yes	0	0.00
	No	30	100.00
Side effects by cosmetics	Yes	0	0.00
	No	30	100.00
Allergy	Food allergy	0	0.00
	Metal allergy	0	0.00
	Photoallergy	0	0.00
	Other allergy	0	0.00
	No	30	100.00
Skin diseases	Acne	0	0.00
	Atopy	0	0.00
	Hair loss	1	3.33
	Extra skin diseases	0	0.00
	No	29	96.67
Tight feeling	Yes	0	0.00
	No	30	100.00
Taking supplements	Taking oriental herb medicines	0	0.00
	Taking nutrients	6	20.00
	Taking extra supplements	0	0.00
	No	24	80.00
Smoking	No	30	100.00
	Less than 10 pieces	0	0.00
	More than 10 pieces	0	0.00
Menstrual cycle	Within 1 wk before menstruation	8	26.67
	During menstruation	3	10.00
	Within 1 wk after menstruation	6	20.00
	The others	13	43.33

product, including toners with no functional ingredients, moisturizers, foundation, powder, cosmetic pencils, and lipstick. Subjects were asked to apply sunscreen products before going outside. The subjects were asked not to apply any colored make-up prior to assessments. The Guidelines for the Evaluation of the Effectiveness of Functional Cosmetic Products (2003, 2005), cosmetics displays, and the Testing Methods Guidelines for the Validation of Ads (2013) were used to select study subjects. The test product contained 0.25% of TA-III (Table 1) was applied to crow's feet region twice a day during the 12 weeks.

TABLE 7 Results of human skin primary irritation test (n = 30)

			48 h				72 h				Reaction grade		
No	Test material	No. of responder	1+	2+	3+	4+	1+	2+	3+	4+	48 h	72 h	Mean
1	TA-III	0	–	–	–	–	–	–	–	–	0.0	0.0	0.0
2	Negative control	0	–	–	–	–	–	–	–	–	0.0	0.0	0.0

TABLE 8 Skin condition of volunteers by skin physiological factors (n = 21)

Items	Classification	Frequency (N)	Percentage (%)
Hydration	Sufficient	0	0.00
	Normal	13	61.90
	Deficient	8	38.10
Sebum	Glossy	1	4.76
	Normal	15	71.43
	Deficient	5	23.81
Surface	Smooth	3	14.29
	Normal	15	71.43
	Rough	3	14.29
Thickness	Thin	3	14.29
	Normal	14	66.67
	Thick	4	19.05
Duration of UV exposure	Less than 1 h	10	47.62
	1-3 h	10	47.62
	More than 3 h	1	4.76
Sleeping hours	Less than 5 h	2	9.52
	5-8 h	17	80.95
	More than 8 h	2	9.52
Smoking	No	21	100.00
	Less than 10 pieces	0	0.00
	More than 10 pieces	0	0.00
Irritability	Yes	1	4.76
	No	20	95.24
Stinging	Yes	0	0.00
	No	21	100.00
Adverse reaction	Yes	0	0.00
	No	21	100.00

2.6.2 | Visual assessment of skin wrinkles

Wrinkles around the eyes were independently assessed under specific lighting conditions. At each assessment, wrinkles were graded into 10 stratified grades in 0.5-point increments; the mean of the two values chosen by the two evaluators was used in the statistical analysis (Table 4).

2.6.3 | Evaluation of skin wrinkle parameters using skin replicas

To evaluate skin wrinkles, skin replica images were analyzed using a Visioline® VL650 (C+K) at each assessment session. This device captures images by transmitting light to the replica at an angle of 35°; it is equipped with a fabricated SILFLO replica that is installed

TABLE 9 Statistical analysis of visual assessment after application of the test and control groups

Group	Week	N	Mean[a]	SD	SEM	*P*-value[b]
TA-III (A)	Before	21	5.21	1.09	0.24	—
	4	21	5.19	1.10	0.24	0.162
	8	21	5.08	1.09	0.24	0.008**
	12	21	4.93	1.03	0.23	0.000***
Control (B)	Before	21	5.10	1.09	0.24	—
	4	21	5.10	1.09	0.24	—
	8	21	5.02	1.07	0.23	0.083
	12	21	4.98	1.03	0.23	0.009**

[a]Decrement of the mean value represents decrease in wrinkle.
[b]Significantly different at $^{**}P < 0.01$, $^{***}P < 0.001$ compared with before treatment.

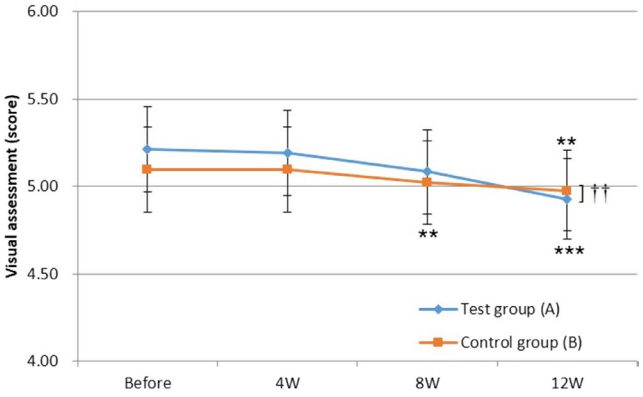


FIGURE 5 Changes in visual assessment following 12 consecutive weeks of application of the product (TA-III) and control groups (mean ± SEM, $^{**}P < 0.01$, $^{***}P < 0.001$ vs before treatment, $^{††}P < 0.01$ vs control group)

in its frame. The images were marked with green shadows and automatically quantified; they were then analyzed for five wrinkle parameters: skin roughness (Rt), maximum roughness (Rm), average roughness (Rz), smoothness depth (Rp), and arithmetic average roughness (Ra; Table 5).

2.6.4 | Subject questionnaire

After 4, 8, and 12 weeks of using the product, subjects were instructed to complete a product efficacy questionnaire that used a 5-point scale: 1 = I do not agree at all, 2 = I do not agree, 3 = There is no difference, 4 = I agree, 5 = I strongly agree. After 12 weeks of using the product, participants completed a product usability questionnaire that also used a 5-point scale: 1 = It is not effective at all; 2 = It is not effective; 3 = It is normal; 4 = It is effective; 5 = It is very effective; the questionnaire was analyzed for positive answers (scores of 4, 5).

2.7 | Statistical analysis

All data were analyzed for statistical significance using the SPSS® Package Program (IBM). For the visual assessment, the evaluators were considered reliable when the intraclass correlation coefficient was >0.8, and the mean value was analyzed. The normality of the distribution was tested using kurtosis and skewness, and prehomogeneity was verified using a paired *t* test. A repeated-measures analysis of variance (ANOVA) was used to determine interdependence (or reciprocal action) between repeated measurements, as well as to compare groups. Statistical analysis was done in terms of comparison between both groups (test vs control group).

The efficacy questionnaire was evaluated using the Mann-Whitney *U* test to compare the two groups in terms of nonparametric mean values; the product usability questionnaire was evaluated using the chi-squared test. Statistical significance was defined as a *P*-value < 0.05.

3 | RESULTS

3.1 | Photoprotective effects of TA-III on HaCaT cells

UVB irradiation reduced cell viability to 49.6% as compared with the control level. However, incubating the cells with 0.1 μmol/L of TA-III prior to irradiation, cell viability was maintained at 100.4% of the control level (Figure 2).

3.2 | MMP-1 inhibition by TA-III

To find out active compound in *A asphodeloides*, extracts and various compounds (timosaponin A-III, timosaponin B, timosaponin B-II, anemarsaponin B, anemarsaponin E, and timosaponin C) from *A asphodeloides* were evaluated in terms of skin wrinkle reduction as measured by MMP-1 level in UVB-treated cells. MMP-1 levels were increased by UVB irradiation (Figure 3). Among them, TA-III showed strong activity on MMP-1 inhibition. Also, TA-III was isolated as major compound in *A asphodeloides*.

3.3 | Effects of TA-III on TIMP and inflammatory cytokines

The mRNA levels of TIMP were measured by RT-PCR after UVB irradiation of HaCaT cells. The level of TIMP was decreased after UVB irradiation, as compared to the control group (Figure 4A). In results of mRNA expression, TA-III increased TIMP mRNA levels as compared with the UVB-irradiated cells.

The expression of pro-inflammatory cytokine mRNA transcripts was investigated in TA-III-treated cells. According to the result in Figure 4(B-D), IL-1β, IL-8, and TNF-α mRNA levels were increased; however, TA-III reversed this phenomenon. This suggested that photoprotective effect of TA-III is based on anti-inflammatory activity on UVB-irradiated cells.

3.4 | Subjects included in the skin safety test

A total of 30 individuals participated in safety test. The mean age of the subjects was 42.1 ± 5.2 years, with the oldest being 50 years old and the youngest being 27 years old. The skin characteristics of the study subjects were examined through surveys, and the results are presented in Table 6. During the study period, no skin reactions to the test solution (TA-III) were observed (Table 7).

3.5 | Skin characteristics of subjects for antiwrinkle test

A total of 23 women aged 43-55 years were included in this test. Two subjects dropped out (No. 4 and No. 12) for personal reasons; the clinical trial was conducted with 21 subjects. After finishing the clinical evaluation, the questionnaire was used to determine the characteristics of the subjects participating in clinical trial as shown in Table 8.

TABLE 10 Statistical analysis of skin wrinkle parameter following application of the test and control groups

Parameters	Group	Week	N	Mean[a]	SD	SEM	*P*-value[b]
Rt	TA-III (A)	Before	21	63.27	9.53	2.08	—
		4	21	62.22	9.61	2.10	0.002**
		8	21	60.82	9.77	2.13	0.000***
		12	21	58.86	9.38	2.05	0.000***
	Control (B)	Before	21	62.73	9.29	2.03	—
		4	21	62.42	9.16	2.00	0.408
		8	21	61.72	9.03	1.97	0.010*
		12	21	60.68	8.63	1.88	0.000***
Rm	TA-III (A)	Before	21	57.68	8.85	1.93	—
		4	21	56.51	8.77	1.91	0.003**
		8	21	55.12	8.85	1.93	0.000***
		12	21	53.02	8.38	1.83	0.000***
	Control (B)	Before	21	57.17	8.48	1.85	—
		4	21	56.58	8.25	1.80	0.294
		8	21	55.79	7.87	1.72	0.016*
		12	21	55.17	7.81	1.71	0.000***
Rz	TA-III (A)	Before	21	42.78	6.80	1.48	—
		4	21	42.09	7.29	1.59	0.104
		8	21	41.07	6.51	1.42	0.000***
		12	21	39.30	6.42	1.40	0.000***
	Control (B)	Before	21	42.48	5.99	1.31	—
		4	21	41.81	6.24	1.36	0.177
		8	21	41.23	6.24	1.36	0.022*
		12	21	40.39	5.85	1.28	0.000***
Rp	TA-III (A)	Before	21	35.54	6.06	1.32	—
		4	21	35.26	6.31	1.38	0.599
		8	21	34.67	6.36	1.39	0.066
		12	21	33.50	6.38	1.39	0.000***
	Control (B)	Before	21	34.92	5.94	1.30	—
		4	21	35.35	5.82	1.27	0.333
		8	21	34.59	5.44	1.19	0.397
		12	21	34.08	5.18	1.13	0.102
Ra	TA-III (A)	Before	21	7.00	1.32	0.29	—
		4	21	6.69	1.42	0.31	0.008**
		8	21	6.62	1.28	0.28	0.003**
		12	21	6.38	1.28	0.28	0.000***
	Control (B)	Before	21	6.69	0.95	0.21	—
		4	21	6.53	0.93	0.20	0.326
		8	21	6.60	1.05	0.23	0.537
		12	21	6.61	1.09	0.24	0.570

[a]Decrement of the mean value represents decrease in wrinkle.
[b]Significantly different at *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with before treatment.

3.6 | Visual assessment

In comparison within groups, wrinkles around the eyes were significantly ameliorated after both 8 and 12 weeks in the test group ($P < 0.01$, Table 9). In the control group, wrinkle reduction was shown after 12 weeks ($P < 0.01$, Table 9). In comparison between groups, the test group (TA-III) showed significant wrinkle reduction after 12 weeks ($P < 0.01$, Figure 5).

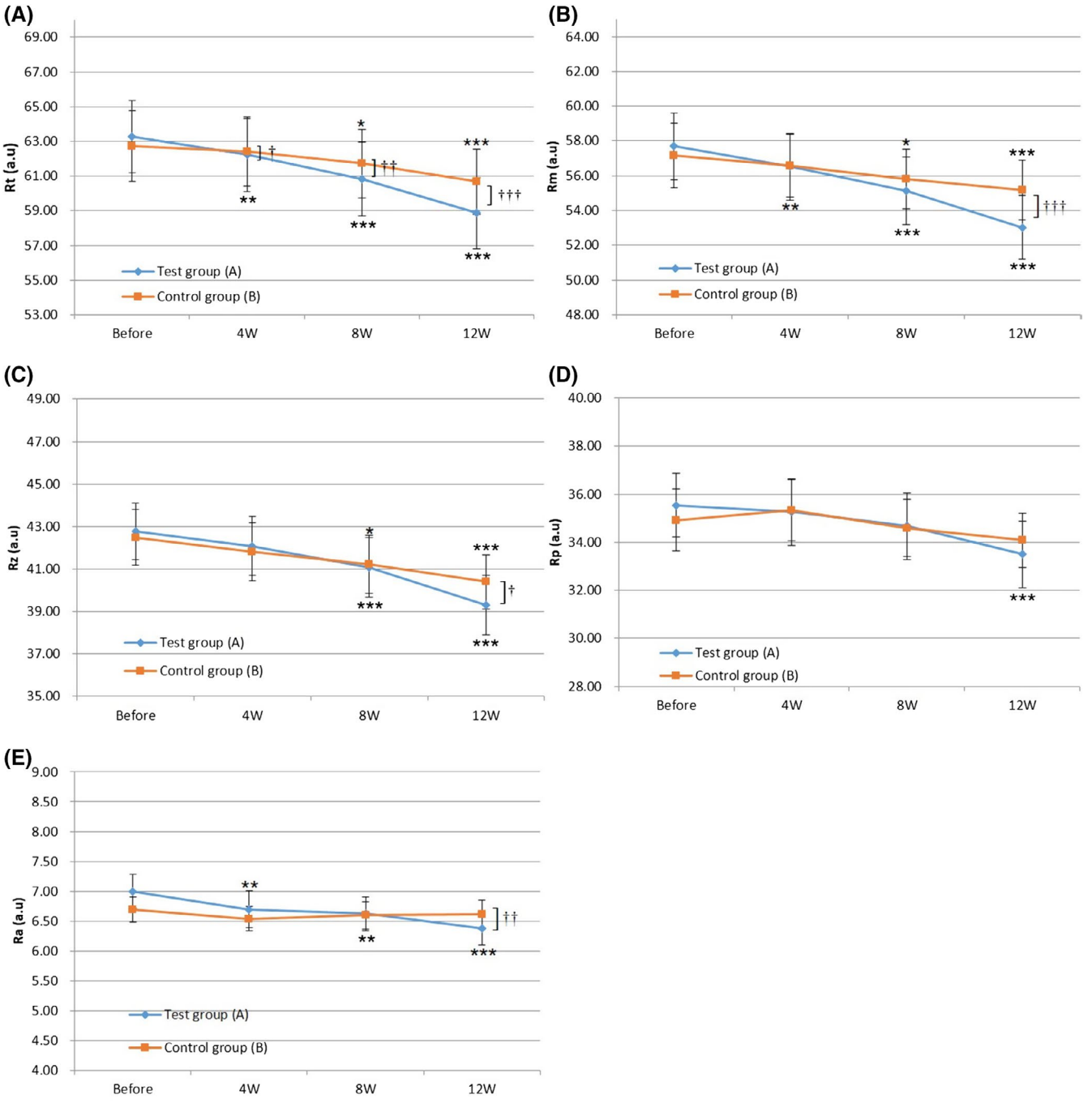


FIGURE 6 Evaluation of skin wrinkle parameters using replica. A, Changes in skin roughness (Rt) following 12 consecutive weeks of application of the test (TA-III) and control groups. B, Changes in maximum roughness (Rm) following 12 consecutive weeks of application of the test (TA-III) and control groups. C, Changes in average roughness (Rz) following 12 consecutive weeks of application of the test (TA-III) and control groups. D, Changes in smoothness depth (Rp) following 12 consecutive weeks of application of the test (TA-III) and control groups. E, Changes in arithmetic average roughness (Ra) following 12 consecutive weeks of application of the test (TA-III) and control groups (mean ± SEM, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs before treatment, $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$, $^{\dagger\dagger\dagger}P < 0.001$ vs control group)

3.7 | Analysis of wrinkle parameters using skin replicas

3.7.1 | Comparisons within groups (4, 8, and 12 weeks)

In the test group (TA-III), Rt, Rm, and Ra were significantly reduced after 4, 8, and 12 weeks of product use, while Rz was significantly reduced after 8 and 12 weeks of product use and Rp was significantly reduced after 12 weeks of product use ($P < 0.01$). In the control group, Rt, Rm, and Rz were significantly reduced after 8 and 12 weeks of product use ($P < 0.05$; Table 10, Figure 6A–E).

TABLE 11 Statistical analysis of skin wrinkle parameter between the test and control groups (*P*-value)

Parameters	Group	4 wk[a]	8 wk[a]	12 wk[a]
Rt	Test vs Control	0.049*	0.006**	0.000***
Rm	Test vs Control	0.308	0.079	0.000***
Rz	Test vs Control	0.974	0.440	0.014*
Rp	Test vs Control	0.202	0.436	0.062
Ra	Test vs Control	0.420	0.128	0.006**

[a]Significantly different at $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ compared with the control group.

3.7.2 | Comparison between groups (control group and test group)

In the test group (TA-III), Rt was significantly improved in the test group compared with the control group after 4, 8, and 12 weeks of product use; Rm, Rz, and Ra were significantly improved in the test group compared with the control group after 12 weeks of product use ($P < 0.05$; Table 11, Figure 6A–E).

3.8 | Subject questionnaire

3.8.1 | Product efficacy questionnaire

Among all participants, 76%-86% of subjects in both the test and control groups responded positively to the items "Increase of skin moisture" and "Improvement of skin softness" after 8 and 12 weeks. Also, 48%-62% of subjects in both the test and control groups responded positively to the item "Improvement of skin glossiness," "Improvement of skin elasticity," and "Decrease of skin wrinkle" after 8 and 12 weeks; this did not constitute a significant difference (Table 12 and Figure 7).

3.8.2 | Product usability questionnaire

In both the test (TA-III) and control groups, 48%-62% and 43%-62% of subjects, respectively, responded positively to the items "color," "viscosity," "absorptivity," and "satisfaction," but there was no significant difference between the groups (Table 13 and Figure 8).

4 | DISCUSSION

Skin aging may result from cumulative exposure to environmental factors related to wrinkle formation.[23] Aging over time is a natural process caused by internal physiological factors that affect the skin similarly to other organs, leading to formation of relatively slight wrinkle.[24] By contrast, photoaging is correlated with exposure to the UV radiation in sunlight, which strongly contributes to a premature aging phenotype.[25]

Skin elasticity is reduced by the extracellular matrix degeneration with aging, and it is the most common feature of skin aging.[26] Wrinkles are formed by the breakdown of extracellular matrix such as collagen and elastin.[27] The complex mechanism such as collagen degradation and inhibition of new collagen production results in wrinkle formation in the dermis.[28]

TABLE 12 Results of positive answers in self-assessment for efficacy (n = 21)

		TA-III group (A)		Control group (B)		
Items	Week	N	%	N	%	*P*-value
Increase of skin moisture	4	11	52.38	8	38.10	0.390
	8	17	80.95	17	80.95	0.781
	12	18	85.71	16	76.19	0.306
Improvement of skin softness	4	15	71.43	11	52.38	0.248
	8	17	80.95	16	76.19	0.743
	12	18	85.71	17	80.95	0.483
Improvement of skin glossiness	4	11	52.38	11	52.38	0.877
	8	11	52.38	11	52.38	0.879
	12	11	52.38	10	47.62	0.672
Improvement of skin elasticity	4	11	52.38	10	47.62	0.672
	8	13	61.90	12	57.14	0.774
	12	12	57.14	12	57.14	1.000
Decrease of skin wrinkle	4	6	28.57	7	33.33	0.742
	8	11	52.38	10	47.62	0.760
	12	11	52.38	12	57.14	0.759

Note: N (Frequency) = Number of positive answers (4, Agree ~ 5, Strongly agree); % (Percentage) = Number of positive answers/Total number of subjects (21) × 100.

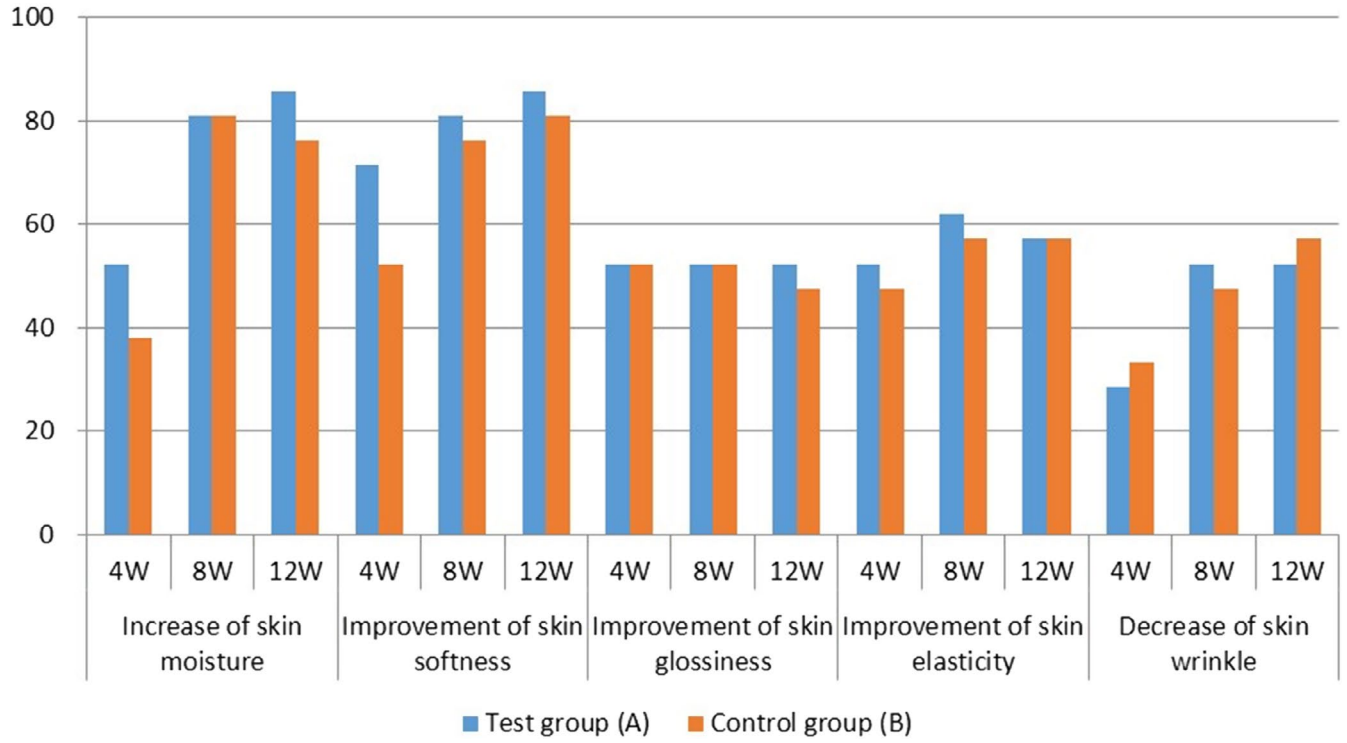


FIGURE 7 Comparative sensorial profile of the test (TA-III) and control groups for efficacy (positive answers, %)

Timosaponin A-III (TA-III) is present as one of the major chemical constituents in *A asphodeloides* rhizomes, and it is reported to have various biological effects.[29,30] In this research, we confirmed the photoprotective activity of TA-III and compounds against UVB damage on skin cells.

MMPs and TIMPs are playing a role in the regulation of collagen metabolism.[31] MMPs produced by UVB exposure cause collagen degradation or inhibition of collagen synthesis, resulting in weak skin connective tissue.[32,33] We have already evaluated similar saponins such as *A asphodeloides* extracts and various compounds (timosaponin A-III, timosaponin B, timosaponin B-II, anemarsaponin B, anemarsaponin E, and timosaponin C). Among them, timosaponin A-III showed strong activity on MMP-1 inhibition. Also, TA-III was isolated as major compound in *A asphodeloides* which means cost effective for cosmetic development. The photoprotective properties of TA-III were measured in terms of the significant reduction in MMP-1 and an increase in TIMP-1.

Keratinocyte induces the NF-κB pathway and inflammatory cytokines when exposed to UVB. These are associated with skin inflammatory responses.[34] Cytokines such as IL-1β are known to stimulate

TABLE 13 Result of positive answers in self-questionnaires for usability (n = 21)

	TA-III group (A)		Control group (B)		
Items	N	%	N	%	*P*-value
Color	13	61.90	10	47.62	0.352
Scent	7	33.33	6	28.57	0.939
Viscosity	10	47.62	9	42.86	0.990
Absorption	12	57.14	9	42.86	0.643
Satisfaction	13	61.90	13	61.90	0.978

Note: N (Frequency) = Number of positive answers (4, Good ~ 5, Very good); % (Percentage) = Number of positive answers/Total number of subjects (21) × 100.

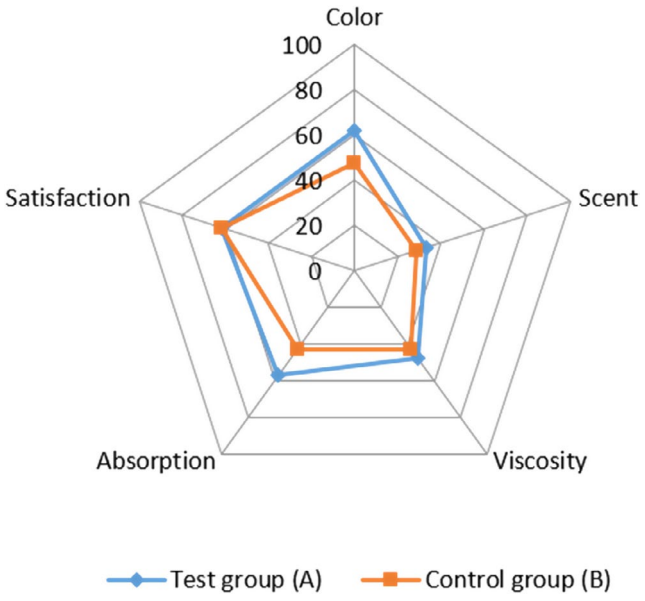


FIGURE 8 Comparative sensorial profile of the test (TA-III) and control groups for usability (positive answers, %)

the expression level of MMP-1 in fibroblasts.[35] The qRT-PCR analyses showed that TA-III attenuated the UVB-induced production of pro-inflammatory cytokine mRNAs in HaCaT cells, including IL-1β, IL-8, and TNF-α. This study demonstrated that exposure to UVB upregulated pro-inflammatory cytokines and MMPs. The expression of inflammatory cytokines was increased by UVB irradiation in HaCaT cells which decreased the cell viability, but this phenomenon was suppressed by TA-III.

In further study of clinical trials, the clinical safety of an agent containing 0.25% of TA-III for use on human skin was performed. In comparison between groups, there was a significant difference in wrinkle parameters measured by replica at different time points. Furthermore, replica analysis in comparison between the control and test groups, Rt was significantly improved in the test group compared with the control group after 4, 8, and 12 weeks of product use; Rm, Rz, and Ra showed significant difference in the test group compared with the control group after 12 weeks of product use ($P < 0.05$).

Safety and toxicity are important considerations for cosmetic products, as application of the product to the skin could lead to systemic exposure, resulting in adverse localized effects such as irritation, sensitization, or photoreactions.[11] Currently, a variety of naturally occurring agents that have antiaging effects are being used in skin care products.[36,37] In general, the effectiveness of cosmetics is assessed by clinical trial experts and wrinkle assessment is considered a primary endpoint.[38,39] According to skin safety results in this study, no dermatological problems were observed in subjects participating in clinical trial. In conclusion, TA-III attenuated UVB-induced skin damage and daily application of TA-III for 12 weeks significantly reduced signs of facial aging by limiting wrinkle formation.

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ETHICAL APPROVAL

In this study, clinical trial was carried out in agreement with the guidelines of Ministry of Food and Drug Safety (MFDS), the United States Personal Care Products Council (PCPC), and the Standard Operating Procedures (SOP) of the DermaPro Institute of Dermatological Sciences (Seoul, South Korea). Furthermore, this study was performed according to the standard operating procedures (SOPs) of the Dermapro Skin Science Institute and it has been uploaded to http://cris.nih.go.kr/cris/index.jsp (identifier KCT0002223) from National Research Institute of Health.

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