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Anti-wrinkle activity of *Platycarya strobilacea* extract and its application as a cosmeceutical ingredient

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Synopsis

In order to investigate the potential of *Platycarya strobilacea* fruit extract as an active ingredient for cosmetics, we measured their free-radical scavenging activity, elastase inhibitory activity, the expression of MMP-1 (matrix metalloproteinase-1), and type I collagen synthesis in normal human fibroblast cells. To isolate the main component compounds from *P. strobilacea* fruit extract, we purified the extract through solvent fractionation, column chromatography, and recrystallization. The component compounds were identified as ellagic acid and 4-*O*-xyloside of ellagic acid (ellagic acid 4-*O*-xylopyranoside). *P. strobilacea* fruit extract and ellagic acid increased the expression of type I collagen mRNA in a dose-dependent manner (up to 37% and 41% at 20 μg/ml and 1.0 μg/ml, respectively), comparable to that of ascorbic acid (up to 39% at 500 μM). A clinical study of measurements using visual evaluation and image analysis showed a statistically significant difference ($p < 0.05$) between the effects of the test and placebo products. This result suggests that *P. strobilacea* fruit extract could be used as an active ingredient for anti-aging cosmetics.

INTRODUCTION

Skin aging is characterized by a progressive deterioration of the skin's functional properties, linked to alterations of dermal connective tissue due to the changes at the cell, gene, and protein levels. Skin aging can be divided into two basic processes: intrinsic aging and photoaging. Intrinsic skin aging describes the irreversible physiological process that starts in age groups from around 17 to 25 years, as soon as physical maturation is accomplished. Photoaging is the term given to the superposition of chronic sun damage on the intrinsic aging process (1–3).

The dermis is the thicker, deeper layer of the skin underlying the epidermis, and is mainly composed of such connective tissues as collagen and elastic fibers. Among these, collagen fiber is the main component of the extracellular matrix (ECM), as the representative connective tissue that comprises about 90% of the dermis; collagen has a direct influence on skin tension. Maintenance of the collagen structure is related to the intrinsic aging and photo-aging processes of the skin (4,5). Therefore, a variety of investigations have been focused on protection against skin aging through the inhibition of collagenase activity, which disintegrates the ECM proteins (6). Matrix metalloproteinases (MMPs) are an enzyme family to digest basal membranes and ECM. MMPs can be divided into four categories according to the preferred substrate: collagenases (MMP-1,8,13,18), gelatinases (MMP-2,9), stromelysins (MMP-3,10), and membrane-type MMPs (MMP-14,15,16,17) (7). It is therefore suggested that an evaluation of the inhibitory efficacy of materials for MMP-1 gene expression could be used as a screening method to uncover promising candidates that would inhibit the degradation of collagens (8–10).

Platycarya strobilacea Sieb. et Zucc. (family: Juglandaceae) is widely distributed throughout Korea, Japan, Taiwan, and China (11). It is commonly called Kool-pee tree in Korea. The root bark of this species have been used in traditional oriental medicine for the treatment of inflammation and diarrhea. Its leaves have also been used for the treatment of acute suppuration or abscess on the skin and head region (12). The constituents of the bark and leaves have been studied. It is known that euglon and tannin exist in the bark and fruit, respectively (13).

We have previously screened various plants for free-radical scavenging activity and the inhibition of elastase activity (14), and have also reported the anti-wrinkle activity of ziyuglycoside I isolated from a *Sanguisorba officinalis* root extract, which showed a significant inhibitory effect against aging (15).

In this study, we investigated free-radical scavenging activity, elastase inhibition activity, reduction of MMP-1 mRNA expression *in vitro*, and type I collagen synthesis in normal human fibroblasts for the development of potential anti-wrinkle ingredients as raw materials for use in cosmetic products. We elucidated the main components from the *P. strobilacea* fruit extract.

MATERIALS AND METHODS

REAGENTS AND EQUIPMENT

All reagents were purchased from Sigma-Aldrich (St. Louis, MO). Other commercially available reagents and solvents were used as received. Human fibroblasts were acquired from ATCC (American Type Culture Collection, CRL-2076). The compound 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was used as received. Medium, fetal bovine serum, and antibiotics were purchased from Life Technologies (Grand Island, NY). The elastase inhibitory activity assay was performed with a UV-spectrophotometer (Hewlett-Packard, HP-8453). An ELISA reader (Tecan, A-5082, Austria) and PCR (Bio-Rad, Mycycler™ thermal cycler) were used in the cytotoxicity assay and in the assay on the inhibition of MMP-1 expression.

EXTRACTION AND FRACTIONATION

P. strobilacea fruits were collected from Jeju island and authenticated by Dr. C. S. Kim, the director of Halla Arboretum in Mt. Halla National Park. The collected fruits were dried in the shade at room temperature and stored in a dark, cold room until needed. The dried whole fruits (180 g) were extracted twice with 70% (v/v) ethanol (20 times as much as the weight of the dried fruits) for 24 h at room temperature. The extract of the fruits was filtered through filter paper (Whatman, No. 5) and then evaporated at 60°C. The filtrate was evaporated to produce the crude *P. strobilacea* fruit extract as a powder (49.5 g, 27.5% yield).

The crude *P. strobilacea* fruit extract (20 g) was dissolved in distilled water at a concentration of 2% (w/w), and the aqueous suspension was successively extracted with n-hexane, ethyl acetate, and n-BuOH. Each fraction was evaporated to dryness to give a residue [The yields were 0.72 g (3.6%) of the hexane fraction; 1.66 g (8.3%) of the EtOAc fraction; 3.0 g (15.1%) of the n-BuOH fraction; and 14.1 g of the H_2O fraction (70.5%).] The n-BuOH fraction (1.0 g) was chromatographed on octadecylsilyl-silica gel (ODS, Capcell Pak C18 UG120, 5 μm, 20 × 250 mm, Shiseido Co., Ltd. Tokyo) by preparative HPLC with isocratic eluent (CH_3CN:H_2O=45:55) at a flux of 14.0 ml/min. Two major compounds on prep-HPLC, ellagic acid and ellagic-4-*O*-xyropyranoside, were isolated (64 mg and 22 mg, respectively) (mp 254–258°C (dec.)) and identified by ^{1}H-NMR and ^{13}C-NMR, the results of which were in agreement with previously published data (12).

FREE-RADICAL SCAVENGING ACTIVITY TEST

The assay for free-radical scavenging capacity was carried out according to the method that has been reported previously by Blois (16). The DPPH (1,2-diphenyl-2-picrylhydrazyl) radical shows a deep violet color due to its unpaired electron, and radical scavenging capacity can be followed spectrophotometrically by the loss of absorbance at 525 nm. In brief, a 0.2 mM DPPH 95% ethanolic solution (1 ml) was added to a sample of the stock (2 ml). Each sample solution was diluted with a 70% ethanolic solution to final concentrations of 10, 5, and 1 μg/ml, and the samples were then agitated. The optical density at 525 nm was measured after 10 min with a UV/Vis spectrophotometer. The free-radical scavenging activity of each sample was calculated according to the following formula:

$$\text{DPPH radical scavenging activity (\%)} = [1-(OD_s - OD_b)/OD_c] \times 100$$

where OD_s is the absorbance of the experimental sample, OD_b is the absorbance of the blank, and OD_c is the absorbance of the control at 525 nm. The results are reported in terms of SC_{50} (the concentration needed to reduce 50% of DPPH). BHT (di-*t*-butyl hydroxy toluene), a representative antioxidant, was used as a control.

ELASTASE INHIBITION ACTIVITY

The elastase activity was evaluated according to the method previously reported by Kraunsoe *et al*. (17). In order to evaluate the inhibition of elastase activity, the amount of

released *p*-nitroaniline, which was hydrolyzed from the substrate, N-succinyl-Ala-Ala-Ala-p-nitroanilide, by elastase, was read with a maximum absorbance at 410 nm. In brief, 1.015 mM of N-succinyl-Ala-Ala-Ala-*p*-nitroanilide was prepared in a 0.1232 M Tris-HCl buffer (pH 8.0), and this solution (1300 μl) was added to the stock sample (100 μl). Each sample solution was diluted to final concentrations of 100, 50, and 10 μg/ml. The solutions were vortexed and preincubated for 10 min at 25°C before 100 μl of the elastase (pancreatic, Type IV, E0258, Sigma, St. Louis, MO, 0.0375 unit/ml) was added. After vortexing, each solution was placed in a water bath for 10 min at 25°C, and the absorbance was measured at 410 nm.

CYTOTOXICITY ASSAY IN A MONOLAYER CULTURE

Evaluation of cytotoxicity was performed by the 3-[4,5-dimethylthiazol]-2,5-diphenyltetrazolium bromide (MTT) assay. The human fibroblast cells were seeded in 24-well plates at a density of 1×10^5 cells per well and cultured at 37°C in 5% CO_2. After one day, a culture medium (Iscove's modified Dulbeco's medium, IMDM) was replaced with a serum-free medium and the cells were incubated in a CO_2 incubator at 37°C in the presence of samples for 24 h, before being treated with 100 μl of 2.5 mg/ml MTT solution. The cells were then incubated at 37°C for an additional 4 h. The medium containing MTT was discarded, and MTT formazan that had been produced by the live cells was extracted with 1 ml of DMSO. The absorbance was read at 570 nm, with a reference wavelength of 650 nm. The cell viability was calculated as follows:

$$\text{Cell viability } (\%) = (OD_{570(\text{sample})}/OD_{570(\text{control})}) \times 100$$

where $OD_{570(\text{sample})}$ is the absorbance of the treated cells at 570 nm and $OD_{570(\text{control})}$ is the absorbance of the negative control at 570 nm (non-treated cells).

ASSAY OF MMP-1 EXPRESSION BY RT-PCR

Human fibroblasts were cultured with IMDM + 10% FBS, 50 U/ml of penicillin, and 50 μg/ml of streptomycin at 37°C in 5% CO_2; the medium was changed every two or three days. When the cells had reached confluence, they were separated by treatment with a 0.25% trypsin–0.03% EDTA (ethylenediamine tetraacetic acid) solution. The cells were seeded into a 60-mm dish at a density of 1×10^6 cells/dish and cultured for one day at 37°C in 5% CO_2. The cultured cells were exposed to UVA (6 J/m^2), and a fresh medium without FBS was added to the cells, which were then treated with samples for 24 h. Total RNA was isolated from the cells with TRIzol (Invitrogen, USA) according to the instructions of the manufacturer. First-strand cDNA synthesis was performed by using random hexamers. The sequences of the primers were as follows: 5′-TGGGAGCAAACA-CATCTGA-3′ (sense) and 5′-ATCACTTCTCCCCGAATCGT-3′ (anti-sense) for MMP-1; 5′-GAGACCTTCAACACCCCAGCC-3′ (sense) and 5′-GGCCATCTCTTGCTCGA-AGTC-3′ (anti-sense) for β-actin. MMP-1 RT-PCR reactions involved reverse transcription at 50°C for 30 min, denaturing at 96°C for 3 min, then 22 cycles of 94°C for 1 min, 48°C for 1 min, and 72°C for 1 min, and finally extension at 72°C for 10 min. The β-actin RT-PCR reactions involved reverse transcription at 50°C for 30 min, denaturing

at 96°C for 3 min, then 25 cycles of 94°C for 1 min, 62°C for 1 min, and 72°C for 1 min, and finally extension at 72°C for 10 min. The final products were detected with 2% agarose gel. The gel was photographed, and the intensity of the stained PCR fragments was quantified from the photographs by a densitometric analysis with Gel Doc 2000 (Bio-Rad Laboratories, Segrate, Milan, Italy). EGCG ((-)epigallocatechin-3-gallate) was used as a positive control.

ASSAY OF TYPE I COLLAGEN MRNA BY RT-PCR

The human fibroblast cells were seeded at a density of 1×10^6 cells/dish in a 60-mm cell culture dish incubated for one day at 37°C in 5% CO_2. The culture medium was replaced with a serum-free medium, and the samples were added to each dish. After 24 h culture, total RNA were isolated with TRIzol reagent and the isolated total RNA were measured at 260 nm. RT-PCR of type I collagen and β-actin were performed using an all-in-one RT-PCR kit (Superbio, Korea) with 1 μg of RNA. The sequences of the primers were as follows: 5′-CTGGCAAAGAAGGCCGCAAA-3′ (sense) and 5′-CTCACCACGATCA-CCACTCT-3′ (anti-sense) for type I collagen mRNA; 5′-GAGACCTTCAACACCCC-AGCC-3′ (sense) and 5′-GGCCATCTCTTGCTCGAAGTC-3′ (anti-sense) for β-actin. Type I collagen mRNA RT-PCR reactions involved reverse transcription at 50°C for 30 min, denaturing at 96°C for 3 min, then 22 cycles of 94°C for 1 min, 62°C for 1 min, and 72°C for 1 min, and finally extension at 72°C for 10 min. The β-actin RT-PCR reactions involved reverse transcription at 50°C for 30 min, denaturing at 96°C for 3 min, then 25 cycles of 94°C for 1 min, 62°C for 1 min, and 72°C for 1 min, and finally extension at 72°C for 10 min. The PCR products were identified by electrophoresis on 2% agarose gel and EtBR (Ethidium bromide, Sigma) staining. The intensity of the stained PCR fragments was also quantified from the photographs by a densitometric analysis with Gel Doc 2000 as above.

ETHICS

This study was conducted according to the guidelines laid down in the Declaration of Helsinki of 1975 as amended in 2000, and all procedures involving human subjects were approved by the Ellead Skin Research Center Institutional Review Board. The volunteers were clearly and precisely informed of the particular objectives and protocol of the study, and of the foreseeable risks involved in the *in vivo* clinical trial. Written informed consent was obtained from all subjects.

IN VIVO CLINICAL TRIAL

Twenty five subjects (34~49-year-old females in good general health) were recruited for this clinical study on a formulation containing *P. strobilacea* fruit extract (the test cream contained 0.2% *P. strobilacea* fruit extract and the placebo did not). This double-blind, placebo-controlled, left-right randomized clinical study was carried out during a 12-week period in order to assess the test and placebo formulations. We measured in this clinical test the efficacy of the formulation in terms of its anti-wrinkling effect on the skin

through a visual evaluation conducted by dermatologists, photometric evaluation, the manufacturing of skin replicas, and image analysis with a Skin-Visiometer SV 600 (Courage & Khazaka, Germany). A cutaneous examination of the crow's feet area was conducted by two dermatologists via a double-blind method. Twenty-five healthy volunteers with dry to very dry skin visited the Ellead Skin Research Center (Republic of Korea) at 0, 4, 8, and 12 weeks, and cutaneous readings were taken according to a photodamage score of 0 to 7 (0, none; 1, none/mild; 2, mild; 3, mild/moderate; 4, moderate; 5, moderate/severe; 6, severe; 7, very severe) (18). We analyzed wrinkling on a monitor by using the 3-Dimensional Skin System program, measuring the number of wrinkle peaks and the depth of each wrinkle. The measuring principle of the Skin-Visiometer SV 600 is based on light transmission through a very thin replica comprising blue-dyed two-component silicone the light absorption of which is known. The roughness parameters investigated were R1 (depth of roughness), R2 (maximum roughness), R3 (mean depth of roughness), R4 (smoothness depth), and R5 (arithmetic average roughness).

STATISTICS

In determining the significance of the data, an independent *t*-test was used to confirm whether the difference was statistically significant (5%). MS EXCEL 2003 software was used for the statistical analysis.

RESULTS AND DISCUSSION

FREE-RADICAL SCAVENGING ACTIVITY

It has been reported that free radicals induced by ultraviolet light or oxidative stress accelerate skin aging (19). Therefore, assays of free-radical scavenging capacity were carried out by the DPPH method. The free-radical scavenging capacity of *P. strobilacea* fruit extract and its fractions was measured at each concentration (1–10 μg/ml), the results being shown in Table I The free-radical scavenging capacity is expressed as SC_{50}, the concentration needed to reduce 50% of the DPPH radical. The crude *P. strobilacea* fruit extract and all the fractions except the hexane fractions (SC_{50} > 10 μg/ml) showed high free-radical scavenging capacity (SC_{50} < 10 μg/ml). Among these, the EtOAc fraction and the BuOH fraction had the highest free-radical scavenging activity (the SC_{50} were 4.9 and 4.8 μg/ml, respectively) compared to BHT (di-*t*-butyl hydroxyl toluene; SC_{50} = 25.4 μg/ml), which was used as a positive control. Ellagic acid especially showed good free-radical scavenging capacity (SC_{50} = 2.5 μg/ml), while ellagic-4-*O*-xyropyranoside did not have DPPH radical scavenging activity. Ellagic acid and ellagic-4-*O*-xyropyranoside isolated from the n-BuOH fraction are known as effective components of *P. strobilacea* (12). Ellagic acid is comprised of four rings of poly phenol, commonly found as a precursor form of ellagic acid, ellagitannin. It is a vegetable phenol found in grapes, strawberries, pomegranates, strawberry trees, peanuts, and green tea and has antioxidant, antiviral, antimutation, and anti-tumor effects. A recent report showed that ellagic acid suppressed the growth of breast, gullet, skin, colon, prostate, and pancreatic cancer cells (13). Ellagic acid is a primary constituent of several tannin-bearing plants that produce the category of tannins known as gallotannins, which give rise to ellagic acid and gallic acid upon hydrolysis by water.

Table I
Free-Radical Scavenging Activity of *P. strobilacea* Fruit Extract

Sample	Fraction	DPPH radical scavenging activity (%)			SC_{50}[b] (μg/ml)
		1 μg/ml	5 μg/ml	10 μg/ml	
P. strobilacea fruit extract	70% EtOH	10.9	50.1	82.2	5.0
	Hexane fr.	2.9	7.8	23	> 10
	EtOAc fr.	13.8	51.2	80.7	4.9
	BuOH fr.	11.1	52.0	80.7	4.8
	H_2O fr.	11.4	48.4	78.6	6.1
Ellagic acid		34.7	81.3	87.8	2.5
BHT[a]		82.5 (100 μg/ml)	64.1 (50 μg/ml)	33.1	25.4

[a]BHT (di-*t*-butyl hydroxyl toluene).
[b]SC_{50} indicates the concentration (μg/ml) at which the percentage inhibition of the DPPH radical scavenging activity was 50%.

INHIBITION OF ELASTASE ACTIVITY

Elastin is the main component of the elastic fibers of the connective tissue and tendons. In the skin, the elastic fibers, together with the collagenous fibers, form a network under the epidermis. Elastase is able to attack all major connective tissue matrix proteins, including elastin, collagen, proteoglycans, and keratins. Because elastic fiber is easily decomposed by elastase secretion and activation caused by exposure to UV light or ROS (reactive oxygen species), an approach that inhibits elastase activity could also be applied as a useful method to protect against skin aging (20). Table II shows the results of the inhibition of elastase activity. The n-BuOH fraction of *P. strobilacea* fruit extract showed the highest elastase inhibition activity (IC_{50} = 35.1 μg/ml). It was over two times higher in effect compared to oleanolic acid (IC_{50} = 83.8 μg/ml), which was used as a positive control. The *P. strobilacea* fruit extract also showed significantly high DPPH radical scavenging activity, as mentioned above. Therefore, this result suggested that a *P. strobilacea* fruit extract would have potential as an anti-wrinkle agent for use in cosmetic products.

CYTOTOXICITY ASSAY IN A MONOLAYER CULTURE

In order to evaluate the cytotoxicity of the *P. strobilacea* fruit extract and ellagic acid *in vitro*, samples were prepared at various concentrations and used to treat human fibroblasts (ATCC, CRL-2076). The results of this evaluation are shown in Figure 1. The *P. strobilacea* fruit extract showed no cytotoxicity compared to that of the positive control, up to the effective concentration for anti-wrinkle activity (less than 50 μg/ml). These findings suggest that *P. strobilacea* fruit extract could be used as an effective and safe active ingredient without any associated cytotoxicity.

INHIBITION OF MMP-1 mRNA EXPRESSION

In order to evaluate the inhibition activity of the degradation of collagen fibers in the skin, we investigated the reduction of MMP-1 expression by *P. strobilacea* fruit extract

Table II
Elastase Inhibition Activity of *P. strobilacea* Fruit Extract

Sample	Fraction	Elastase inhibition activity (%) 100 μg/ml	50 μg/ml	10 μg/ml	IC_{50}[a] (μg/ml)
P. strobilacea fruit extract	70% EtOH	72.8	63.7	12.1	37.9
	Hexane fr.	—	—	—	—
	EtOAc fr.	74.3	69.2	7.8	35.1
	BuOH fr.	82.2	67.5	6.1	37.0
	H_2O fr.	56.2	27.6	4.6	89.2
Ellagic acid		6.2 (1 μg/ml)	57.3 (5 μg/ml)	80.6	4.6
Oleanolic acid		57.7	35.2	9.9	83.8

[a]IC_{50} indicates the concentration (μg/ml) at which the percentage inhibition of elastase activity was 50%.

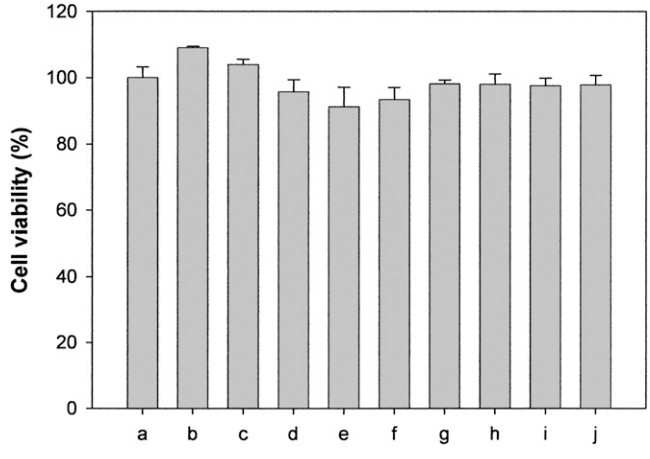

Figure 1. Cytotoxicity assay of *P. strobilacea* fruit extract, ellagic acid, and ellagic acid 4-*O*-xylopyranoside in human fibroblast cells: (a) Control; (b) 10 μg/ml *P. strobilacea* fruit extract; (c) 20 μg/ml *P. strobilacea* fruit extract; (d) 50 μg/ml *P. strobilacea* fruit extract; (e) 0.25 μg/ml ellagic acid; (f) 0.5 μg/ml ellagic acid; (g) 1.0 μg/ml ellagic acid; (h) 0.25 μg/ml ellagic acid 4-*O*-xylopyranoside; (i) 0.5 μg/ml ellagic acid 4-*O*-xylopyranoside; (j) 1.0 μg/ml ellagic acid 4-*O*-xylopyranoside. The data are expressed as mean values (± standard deviations) of five experiments.

and ellagic acid by using the RT-PCR method. EGCG was used as a positive control because its activities are well known to have an inhibitory effect on collagenase and stromelysin mRNA expression induced by IL-1β (21) and to have a protective effect against skin damage caused by UV rays (22). The MMP-1 expression assay on human fibroblasts was carried out with a Gel Doc 2000 image analyzer (Bio-Rad). The *P. strobilacea* fruit extract reduced the expression of MMP-1 (by up to about 76% at 50 μg/ml), as shown in Figure 2. The *P. strobilacea* fruit extract inhibited the expression of MMP-1 from 17.3% to 76.0% at concentrations of 25–50 μg/ml in a dose-dependent manner. But ellagic acid isolated from *P. strobilacea* showed only 15% of inhibition at its maximum concentration (1μg/ml).

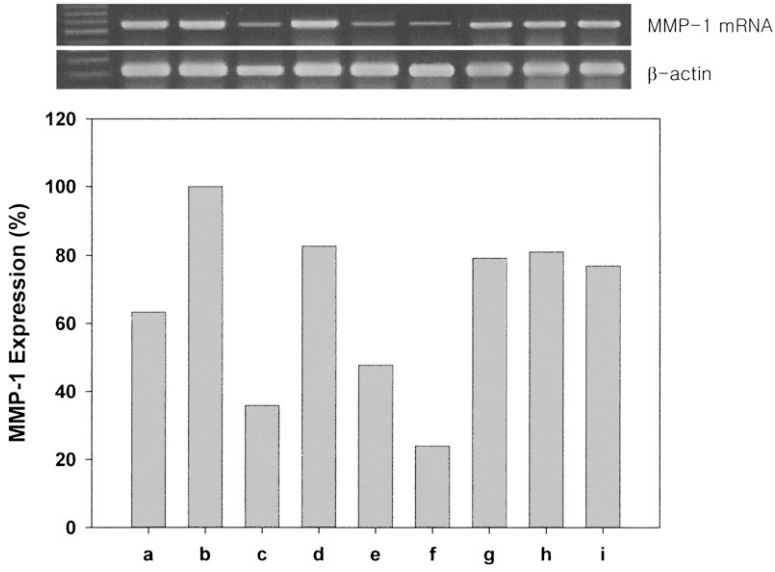

Figure 2. Expression of MMP-1 mRNA in human fibroblasts (ATCC, CRL-2076) by RT-PCR: (a) Control; (b) UVA 6J; (c) UVA 6J + 10 μg/ml EGCG, (-)epigallocatechin-3-gallate; (d) UVA 6J + 10 μg/ml *P. strobilacea* fruit extract; (e) UVA 6J + 20 μg/ml *P. strobilacea* fruit extract; (f) UVA 6J + 50 μg/ml *P. strobilacea* fruit extract; (g) UVA 6J + 0.25 μg/ml ellagic acid; (h) UVA 6J + 0.5 μg/ml ellagic acid; (i) UVA 6J + 1.0 μg/ml ellagic acid.

ASSAY OF COLLAGEN TYPE I mRNA BY RT-PCR

Collagen fiber is the main component of the extracellular matrix (ECM), as the representative connective tissue that comprises about 90% of the dermis. Therefore, collagen has a direct influence on skin tension. To evaluate the amount of collagen type I synthesis that occurred upon exposure to the extract, collagen type I mRNA was quantitatively measured by RT-PCR. *P. strobilacea* fruit extract increased the expression of collagen type I mRNA about 30%, irrespective of concentrations of 25–50 μg/ml. Ellagic acid isolated from the *P. strobilacea* fruit extract especially increased the expression of collagen type I mRNA in a dose-dependent manner (up to 41.3% at 1 μg/ml), comparable to that of ascorbic acid (up to 39.5% at 500 μM), as shown in Figure 3.

IN VIVO CLINICAL TRIAL

We measured the efficacy of the anti-wrinkling effect on the skin in this clinical test through a visual evaluation by dermatologists, photometric evaluation, the manufacturing of skin replicas, and image analysis using the Skin-Visiometer SV 600. The cutaneous evaluation was performed on volunteers during scheduled visits (0, 4, 8 and 12 weeks). The cutaneous readings were based on a photodamage score of 0 to 7 (0, none; 1, none/mild; 2, mild; 3, mild/moderate; 4, moderate; 5, moderate/severe; 6, severe; 7, very severe) and evaluated by two dermatologists (18). The results show that the difference between the test group and the placebo group was not significant until four and eight weeks after the treatment, but that there was a significant difference 12 weeks after the

treatment (Figure 4). In an image analysis of skin replicas by the Skin-Visiometer SV 600, among the roughness parameters already mentioned, skin roughness R1 is the distance between the basic and reference profile, referred to a given reference length L. R3,

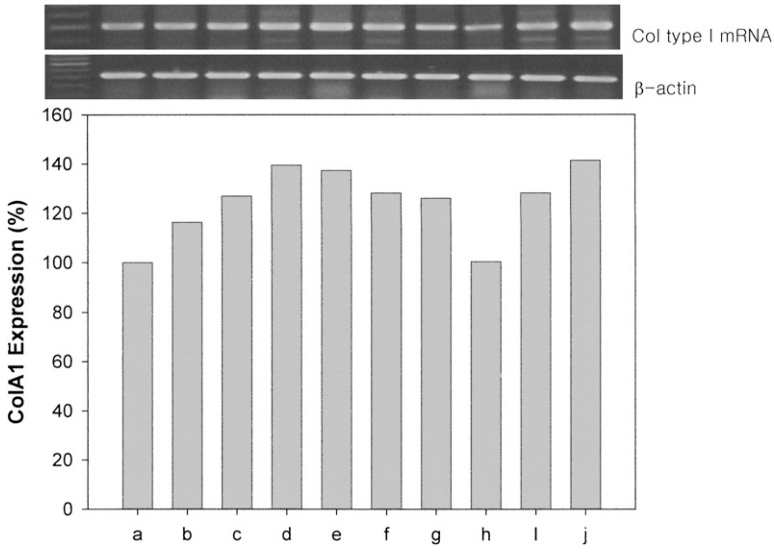

Figure 3. Expression of collagen type I mRNA in human fibroblast cells: (a) Control; (b) 100 μM vitamin C; (c) 250 μM vitamin C; (d) 500 μM vitamin C; (e) 10 μg/ml *P. strobilacea* fruit extract; (f) 20 μg/ml *P. strobilacea* fruit extract; (g) 50 μg/ml *P. strobilacea* fruit extract; (h) 0.25 μg/ml ellagic acid; (i) 0.5 μg/ml ellagic acid; (j) 1.0 μg/ml ellagic acid. The data are expressed as the mean values (± standard deviation) of three experiments.

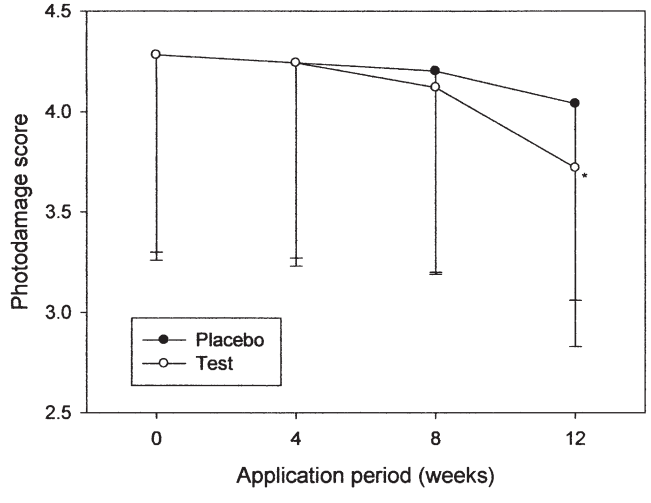

Figure 4. Weekly comparison of photodamage scores in the crow's feet area after the use of the formulation containing *P. strobilacea* fruit extract for 12 weeks (*$p < 0.05$). The visual evaluation was conducted by two dermatologists via a double-blind method. In any case of the readings of the two doctors not agreeing, the lower score for the anti-wrinkle activity was selected for analytical purposes.

the average roughness, is the arithmetic average of the different segment roughnesses. Inherent in their definitions, R3 is the most adequate parameter for studying. The test formulation also showed a significant improvement in the average difference in roughness (ΔR3, -0.02 ± 0.06) than did the placebo formulation (ΔR3, 0.01 ± 0.05) 12 weeks after the treatment (Figure 5). In the clinical study of measurements using visual evaluation and image analysis, the test cream showed a significantly different effect ($p < 0.05$) from that of the placebo (Figure 6).

CONCLUSIONS

In this study, in order to investigate the potential of *P. strobilacea* fruit extract as an active ingredient for wrinkle-care cosmetics, we measured their free-radical scavenging activity, elastase inhibitory activity, the expression of MMP-1 *in vitro*, and type I collagen synthesis in normal human fibroblast cells. *P. strobilacea* fruit extracts ($SC_{50} < 5$ μg/ml) showed very high free-radical scavenging activity compared to BHT ($SC_{50} = 25.4$ μg/ml) as a positive control. Elastase inhibitory activity of *P. strobilacea* fruit extract ($IC_{50} = 37.9$ μg/ml)

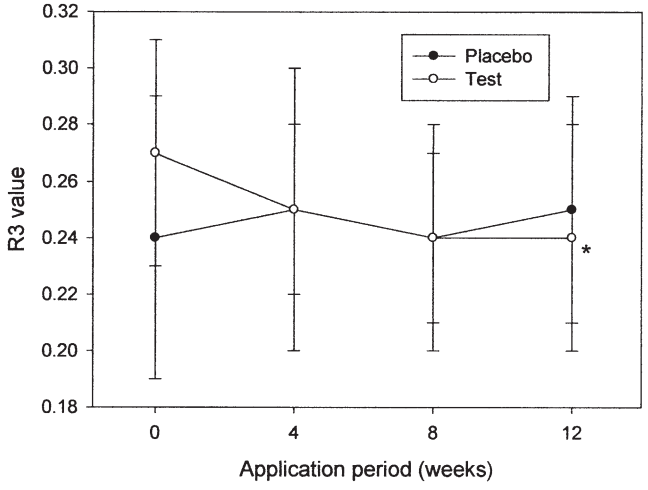

Figure 5. Weekly comparison of R3 (average roughness) values measured by the Skin-Visiometer SV 600 (Courage & Khazaka, Germany) in the crow's feet area after the use of the formulation containing *P. strobilacea* fruit extract for 12 weeks (*$p < 0.05$).

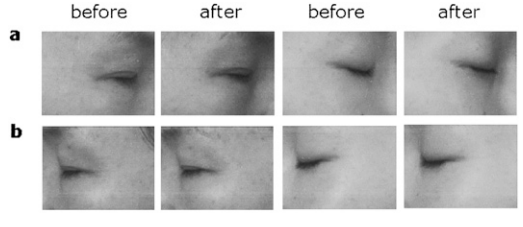

Figure 6. Photographic assessment of wrinkles in the crow's feet area of (a) the test group and (b) the placebo group by CCD camera after the use of the formulation containing *P. strobilacea* fruit extract for 12 weeks.

was over two times higher than that of oleanolic acid (IC_{50} = 83.8 μg/ml). *P. strobilacea* fruit extract reduced the expression of MMP-1 mRNA (up to about 70% at 50 μg/ml). Ellagic acid was isolated as a main component and identified using spectroscopic analysis. In the MMP-1 mRNA expression assay on human fibroblast cells stimulated by UVA radiation, ellagic acid slightly reduced the expression of MMP-1 mRNA about 15% at 1 μg/ml while *P. strobilacea* fruit extract reduced it about 70% at 50 μg/ml. *P. strobilacea* fruit extract and ellagic acid increased the expression of type I collagen mRNA in a dose-dependent manner (up to 37% and 41% at 20 μg/ml and 1.0 μg/ml, respectively), comparable to that of ascorbic acid (up to 39% at 500 μM). *P. strobilacea* fruit extract showed no cytotoxicity up to the effective concentration for anti-wrinkle activity. In this clinical study, the test and placebo formulation (the test cream contained 0.2% *P. strobilacea* fruit extract and the placebo did not) were compared. The visual evaluation and image analysis used in this study showed a statistically significant difference ($p < 0.05$) between the effects of the tested formulation and the placebo 12 weeks after the treatment. All of these results suggest that *Platycarya strobilacea* fruit extract could be used as an active ingredient for new anti-wrinkle cosmetics.

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