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Areca catechu L. extract. I. Effects on elastase and aging

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Synopsis

The anti-aging effects of *Areca catechu* L. extract (CC-516) on skin were investigated both *in vitro* and *in vivo*. The CC-516 is composed of a relatively high amount of protein (26%) and carbohydrate (37.5%), and it has a high proportion of proline (13%) of free amino acid content. The inhibitory effect of CC-516 on the elastase exhibited a 37% to 98% inhibition for a 10–500 μg/ml concentration, and IC_{50} values of 40.8 μg/ml for porcine pancreatic elastase (PPE) and 48.1 μg/ml for human leukocyte elastase (HLE), respectively. One of the effects of elastase is that it is known to reduce the number of elastin fibers at the level of enzyme deposition. The number of elastin fibers was increased when we drifted from the deposit point of elastase with 100 μg/ml of CC-516. CC-516 showed protection of elastic fiber against degradation by the enzyme in an *ex vivo* assay. The CC-516 increased proliferation of human fibroblast cells by 85% at 10^{-4}% concentration, compared with control, whereas the increase with ascorbic acid was 50%. The monolayer and three-dimensional collagen synthesis was increased by 50% at 10^{-4}% and 20% at 0.1% concentrations of CC-516, respectively. The treatment with CC-516 improved skin hydration, skin elasticity, and wrinkle reduction. From this study, we suggest that CC-516 can be used as a new anti-aging component in cosmetics.

INTRODUCTION

With aging, especially of people over 40 years, the elasticity of skin is significantly decreased by elastase activity, which results in sagging (1). Histological examination reveals a thickened epidermis and dermis, an increased number of mast cells and hypertropic cysts, the infiltration of inflammatory cells, the partial absence and aggregation of elastin fibers, and a decrease in collagen fibers. Biologically, elastase activity significantly increases with age (2). The increase results in reduced skin elasticity and in the appearance of wrinkles or stretch marks (3). A number of studies have been interested in interactions between elastase and its inhibitors, which include unsaturated fatty acids, peptides, flavonoids, and terpenoids (4–10). It has been proposed but not yet fully demonstrated (11) that the beneficial effects of these inhibitors on the typical signs of cellulitis are obtained by inhibition of elastase, the lysosomal enzyme which regulates the turnover of the structural constituents of the extravascular matrix that surrounds the capillary walls (12–15). Plant sources have been evaluated for developing natural active

agents that may be involved in anti-aging and anti-wrinkle care (16–17). To develop active agents for skin anti-aging, we previously screened the inhibitory effects of 150 medicinal plants on elastase activity. The *Areca catechu* metanolic extract showed high inhibitory effect on elastase compared with reference compounds (18), and we selected the *Areca catechu* extract as a new candidate for anti-aging agent in cosmetics. *Areca catechu* L. is widely cultivated, especially in southern Asia, and its seed is used as a chewing material, as an anthelmintic, and also as a kompo-traditional medicine. Preparations containing *Areca* are used also as digestive medicine since *Areca* promotes the secretion of saliva (19–20). *Areca catechu* L. contains a number of chemical components, such as alkaloids, tannins, flavonoids, and fatty acids (21,22). These compounds have been tested for their individual mutagenicity in bacterial and some rodent test systems (23).

To clarify the mechanism of *Areca catechu* L. extract against anti-aging, we have studied the inhibitory capacity of elastase *in vitro* and also performed *ex vivo* assessments using human skin tissue and automated image analysis. Furthermore, we investigate here the effects of the *Areca catechu* extract on collagen synthesis, extracellular matrix deposition of human dermal fibroblasts in culture, wrinkle reduction, skin moisturizing, and skin elasticity *in vivo.*

MATERIALS AND METHODS

CHEMICALS

All solvents were of analytical grade. Human leukocyte and porcine pancreatic elastase, ascorbic acid, reference substances, and the basic fibroblast growth factors were purchased from Sigma Chemical Co. (St. Louis, MO). [N-Succ-(Ala)3-*p*-nitroanilide and Meo-Succ-(Ala)2-Pro-Val-*p*-nitroanilide were purchased from Calbiochem. Other reagents were analytical grade from commercial sources.

EQUIPMENT

Spectrophotometric measurements were performed with a UV-visible spectrophotometer (Beckman). The composition analyses of CC-516 were performed with a GC/MS (HP 5890 GC/HP 5970 MS), an amino acid analyzer (TCX 3100, ACS, UK) a TLC (Kiesel gel 60, F_{254} glass plate, Merck), and an HPLC (Shimatzu LC-9A, Japan). The anti-aging effects of the cream containing CC-516 were measured by a corneometer CM 820 (Courage + Khazak, C+K, Germany), a cutometer SEM 474 (C+K Electronic GmbH, Germany), a skin visiometer SV 400 (C+K Electronic GmbH, Germany), an automated image analyzer (BIOCOM 200, France), and a scanning electron microscope (JSM-840 A, Jeol Co.).

PREPARATION OF CC-516

Areca catechu seed was purchased from the oriental medicinal market, Seoul, South Korea, and it was sliced and weighed. 100 g of *Areca catechu* was soaked in 500 ml of 90% ethanolic aqueous solution at room temperature for seven days. After filtration, the ethanolic filtrate was evaporated to dryness under vacuum, and then completely dried by

lyophilization. The dried extract was designated the "CC-516," and used as the sample in this study. *Areca catechu* seed was extracted with other solvents (methanol, ethanol, ether, hexane, chloroform, ethyl acetate, and butanol) by the same extraction process as for the CC-516, and the inhibitory effect on elastase was assayed. Especially, the ethanolic extract showed a high inhibitory effect on elastase. Thus CC-516 was used as the sample in this study.

DETERMINATION OF ELASTIC PROPERTIES

Assay for elastase activity. Porcine pancreatic elastase (PPE; Sigma, Type IV) was assayed spectrophotometrically by the method of James *et al.* (27), using N-Succ-$(Ala)_3$-nitroanilide (S.A.N.A.) as the substrate, and by monitoring the release of *p*-nitroaniline for 20 min at 25°C. The amount of *p*-nitroaniline was determined by measuring the absorbance at 410 nm. The reaction mixture contained 0.2 M Tris-HCl buffer (pH 8.0), 1 μg/ml elastase, 0.8 mM Succinyl-Ala-Ala-Pro-*p*-nitroanilide (ESIV; elastase substrate IV, Calbiochem) as substrate, and CC-516 dissolved in methanol. The CC-516 was preincubated for 20 min at 25°C. The reaction was started by adding the substrate. Blanks contained all the components except the enzyme. Human leukocyte elastase (HLE, Sigma) activity was spectrophotometrically determined by measuring the amount of *p*-nitroaniline at 410 nm for 20 min at 25°C. The reaction mixture contained 0.1 M HEPES buffer (pH 7.5); 1 μg/ml elastase; 0.5 M NaCl; 9.8% DMSO, 1% (v/v); 10 mg/ml BSA (Sigma, fraction V); 1.12 mM Meo-Succinyl-Ala-Ala-Pro-Val-*p*-nitroaniline (ESI, elastase substrate I, Calbiochem) as a substrate; and CC-516. The CC-516 dissolved in methanol was preincubated for 20 min at 25°C, and the reaction was started by adding the substrate. The rate of the reaction was determined by the slope of the line recorded and was proportional to elastase activity. A control curve was prepared with elastase in the absence of CC-516. One unit of elastolytic activity is defined as the activity releasing 1 uM of *p*-nitroaniline/min. The percentage of inhibition was calculated as: Inhibition (%) = $(1 - B/A) \times 100$, where A is the enzyme activity without CC-516 and B is the activity in the presence of CC-516.

Assay for elastase activity on dermal sections. The elastase activity on dermal sections was assayed by the modified method of Donald *et al.* (28). In this study, CC-516 was diluted in 0.1 M HEPES buffer (pH 7.5) containing 0.1 M NaCl. Skin sections were obtained from plastic surgery on the mammary gland of a woman. The plastic surgery cut the mammary gland into fragments of 1 cm on each side. Fragments were placed on a cork support and frozen in liquid nitrogen. Cross sections carried out with a freezing microtome were placed on glass plates and maintained hydrated with the HEPES buffer. The CC-516 dilutions were deposited on skin sections, at the rate of 100 ul per section, and incubated for 30 min at 37°C. After 30 min, an enzyme-impregnated thread (HLE, 1 μg/ml) was deposited on the section. After three hours of incubation, sections were rinsed with HEPES and colored with orcein. The effect of the CC-516 on the enzyme activity was assayed by observing sections by a semi-automated image analysis (section photos were taken), and the sections were graded.

ASSAY FOR ANTI-AGING EFFECTS WITH FIBROBLAST *IN VITRO*

Fibroblast cultures were initiated from biopsies of normal human skin. Tissue was minced and plated onto 75-T plastic tissue flasks. Cells were maintained in Dulbecco's

modified Eagle's medium (DMEM) containing 0.48 mg/ml glutamine, 100 IU/ml penicillin, 50 mg/ml streptomycin, and 10% fetal bovine serum (FBS, Gibco BRL) at 37°C in a 5% CO_2 humidified atmosphere. The cells were obtained after the dermis was separated by trypsine. They were then employed upon the fourth passage and mixed for the purpose of testing.

Preparation of three-dimensional dermal equivalents system. Three-dimensional dermal equivalents were prepared with a 24-well multichamber plate in the Coulomb B method (29) by mixing type I collagen gel, reconstituted buffer (2.2 g $NaHCO_3$, 4.77 g HEPES made up to 1000 ml 0.06N NaOH), and fibroblasts. The collagen matrix contracted during one day due to an active organization of collagen fibrils by the fibroblasts. After one day, three-dimensional dermal equivalents were maintained as described monolayer cell cultures.

Cell proliferation. CC-516 was added into the culture system containing DMEM supplemented with 10% FBS, and the cell proliferation was measured by an MTT assay (30).

Collagen synthesis. The activity of CC-516 on the monolayer and three-dimensional culture was measured by the [H^3]-proline incorporation method (31). Cells cultured onto 24-well multichamber plates were assayed for confluence. The culture medium was changed to Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS containing 20 mCi/ml L[2,3,4,5-H^3]-proline (NEN Chemicals) and incubated at 37°C in 5% CO_2 incubator for 24 hr. The protein concentrations per well were assayed with the protein assay kit (Bio-Rad). After labeling, protease inhibitors were added to the cultures. The media and the cells were mixed and then sonicated. Collagen synthesis was assayed by measuring the radioactivity of the media and cells together after limited degradation with purified bacterial collagenase, according to the method of Peterkofsky *et al.* (32).

The relative rate of collagen synthesis to total protein synthesis was calculated with the assumption that collagen had an amino acid content 5.4 times higher than that of other proteins (33). Collagen synthesis of three-dimensional dermal equivalents was measured in the same manner as the above procedures.

ASSAY OF IMPROVEMENTS OF SKIN CONDITION

The improvements in skin condition were evaluated for three aspects: moisture content of the skin, skin elastic properties, and wrinkles. The instruments used were the corneometer CM 820 (Courage + Khazaka, C+K, Germany) for moisture content of the skin, the cutometer SEM 474 (C+K, Germany) for skin elasticity, and the skin visiometer SV 400 (C+K, Germany) for wrinkles. All the percentage values were calculated as: (%) = (value at measuring point − value at initial point)/value at initial point × 100), and the statistical significance of the two test groups was verified by a paired t-test at the significant level, $p < 0.05$. Evaluation of skin moisturizing efficiency was performed by measuring stratum corneum hydration by the capacitance method (34,35). The long-term (six weeks) effects of CC-516 were topically assessed by twenty healthy volunteers with dry to very-dry skin. After application twice daily to cheek and eye regions, measurements were taken before the first treatment and one week, two weeks, four weeks and six weeks after treatment. In order to minimize the risk of treatment errors we routinely limit the number of treatment areas to two groups (cream containing

3% CC-516, untreated cream as control), and we even prefer the simple left-right control lateral comparison design (36). The cutometer SEM 575 (C+K Electronic GmbH, Germany), which is used for skin elasticity measurements, is based on the suction method. Negative pressure is created in the device, which can be regulated between 20 and 500 mbar. Skin is drawn into the aperture by negative pressure where the skin penetration depth (=ds) is determined by a new non-contact optical measuring system. The optical measuring system consists of a height transmitter and a height recipient. The light intensity will vary due to the penetration depth of the skin. This means the smaller penetration depth, the greater the elasticity. To measure wrinkles by using the image analyzer, we analyzed wrinkles on the monitor by a three-dimensional skin system program, and then measured the number of wrinkle peaks and the depth of the wrinkles. The measuring principle of the skin visiometer SV 400 is based on transmission of a very thin and specially dyed silicon replica. The wrinkles were perpendicular to the axis of the light beam, and measurements of the number (N) and depth (P) of wrinkles were recorded in this position by the program. Test sites were taken under the eyes and forearm of twelve volunteers. The test sample (cream containing 3% CC-516) was applied to the volunteers twice a day for six weeks on designated test sites (37,38). SEM examination was performed on dyed silicon replicas of the tested areas that had been air-dried, coated with a thin layer of gold-palladium, and viewed in a scanning electron microscope (JSM-840A, Jeol Co.) at 25 kV.

RESULTS AND DISCUSSION

We previously screened the inhibitory effect on elastase from methanolic extracts of 150 medicinal plants. The *Areca catechu* extract showed a high inhibitory effect comparable to reference compounds (18). For the inhibitory effect of several solvent extracts on elastase, the ethanolic extract, CC-516, was highest and used as the sample in this study. Table I shows the composition of CC-516, which contains relatively high amounts of protein (26%, w/w) and carbohydrate (37.5%, w/w), and low amounts of lipid (2.8%, w/w). Figure 1 represents the composition and content of amino acids. The presence of alanine, glycine, and proline in CC-516 is worth something, since a basic collagen contains mainly glycine, proline, and alanine. The proline and lysine of amino acids are the biosynthetic precursors to collagen. Table II lists the composition and contents of fatty acids of the hexane fraction. The analysis revealed that the fraction consisted of lauric, myristic, palmitic, stearic, oleic, linoleic, and other acids. Myristic (33.3%) and

Table I
Composition of CC-516

Composition	Protein (1)	Carbohydrate (2)	Lipid (3)	Flavonoid (4)	Ash (5)
(mg/g)	260	375	34	86	9

(1) Protein concentrations were measured by the Lowry assay (24).
(2) The amount of carbohydrate was determined by the phenol-sulfuric assay (25).
(3) Total lipid contents were determined by the hexane extraction method.
(4) Total flavonoid contents were carried out by using the photometric method of 280-nm absorption detection (26).
(5) Ash contents were determined by heating in a muffle furnace (600°C, 6 hr).

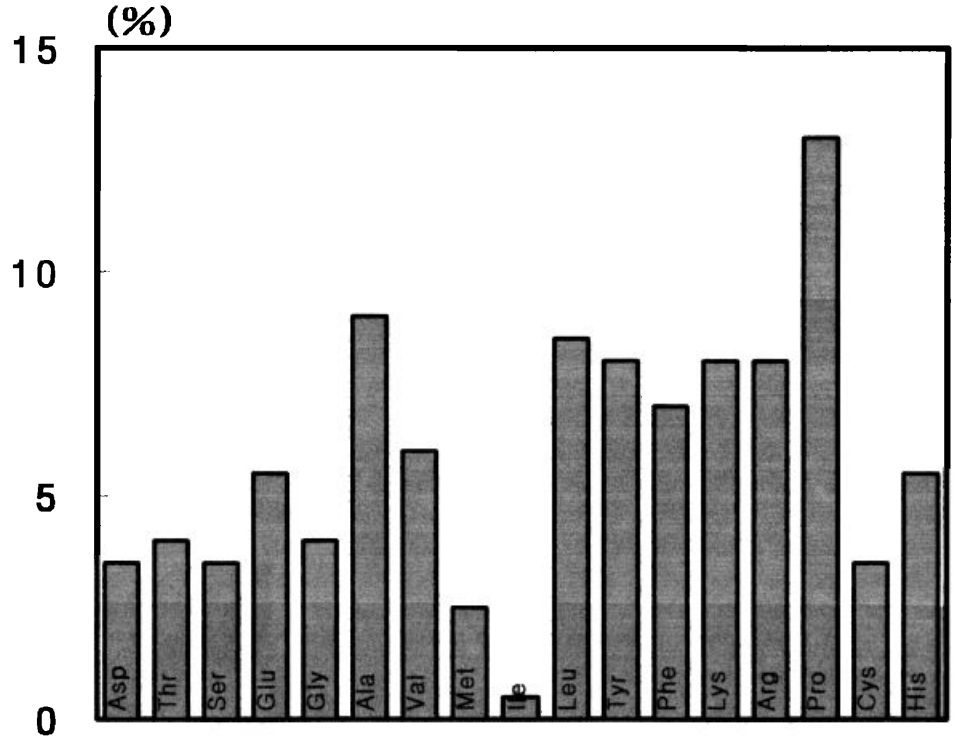

Figure 1. Percentage of total amino acids in CC-516. Free amino acid content was 0.2% determined by amino acid analyzer (TCX 3100, ACS, UK).

Table II
Composition Analysis of Fatty Acids in CC-516 by GC/MS

	Fatty acid						
Composition	Lauric (12:0)	Myristic (14:0)	Palmitic (16:0)	Stearic (18:0)	Oleic (18:1)	Linoleic (18:2)	Others
(%)	24.2	33.3	11.5	1.3	14.4	14.3	1.0

Derivated trimethylsilyl was prepared from the dried extracts (1 mg) by dissolution in Bis(trimethylsily)trifluroacetamide (50 µl) and pyridine (50 µl) heated at 60°C for 30 min. Aliquots of 0.05 µl were injected onto the GC. CC-516 and standards were run alternately on the GC/MS with blanks of BSTFA/pyridine. A fused HP ultra-2 column (Econocap: 50m; capillary: 0.2 mm, thickness: 0.11 µm) was crosslinked with a PMS. The bonded phase was eluted with He (inlet pressure 6 psi) directly into the ion source of a HP 5890 GC/HP 5970 MS. The column was temperature programmed from 50°C to 300°C at 10°C/min. The major components and many of the minor components were identified, after trimethylsilylation, by comparison of their electron impact mass spectra and GC elution times with standard sample spectra.

lauric (24.2%) acids were the major fatty acids of the hexane-soluble fraction. Oleic (14.4%) and linoleic (14.3%) acids appear to be major contents of unsaturated fatty acids. Intercellular lipids, particularly ceramides, play an important role in regulating skin barrier function as well as in maintaining the water-holding capacity of the stratum corneum. Several lipidic substances could inhibit leukocytic elastase, pancreatic elastase,

and plasmin. In particular, their active sites can accommodate unsaturated fatty acids and fatty acid derivatives. It was therefore postulated that CC-516, composed of fatty acids, many of them amino acids, polypeptides and flavonoids, could inhibit HLE and thus attenuate HLE-catalyzed extracellular matrix alterations during skin inflammatory disorders.

Figure 2 shows the concentration-dependent inhibition of PPE and HLE by CC-516. The CC-516 at 10–500 μg/ml as the final concentration exhibited 37% to 98% inhibition, and IC_{50} values were 40.8 μg/ml (PPE) and 48.1 μg/ml (HLE), respectively. Figures 3–5 show the effects of the CC-516 on the enzyme activity *in vivo*. The elastase affects a reduction in the number of elastin fibers at the level of the enzyme deposit. The number of fibers increased when we drifted from the enzyme deposit point. CC-516 has insignificant inhibitory effects at the concentrations of 10 μg/ml and 50 μg/ml. On the other hand, it reduces strongly the elastase activity and thus increases the number of fibers at 100 μg/ml, as shown in Figure 5. This highly protective behavior towards elastin degradation contrasts with the properties of other elastase inhibitors (6). CC-516 could protect elastic fibers against elastase degradation in an *ex vivo* assay. Table III shows the effect of CC-516 on the cell proliferation of human fibroblasts. CC-516 increased cell proliferation by 85% at 10^{-4}% concentration, compared to that of the

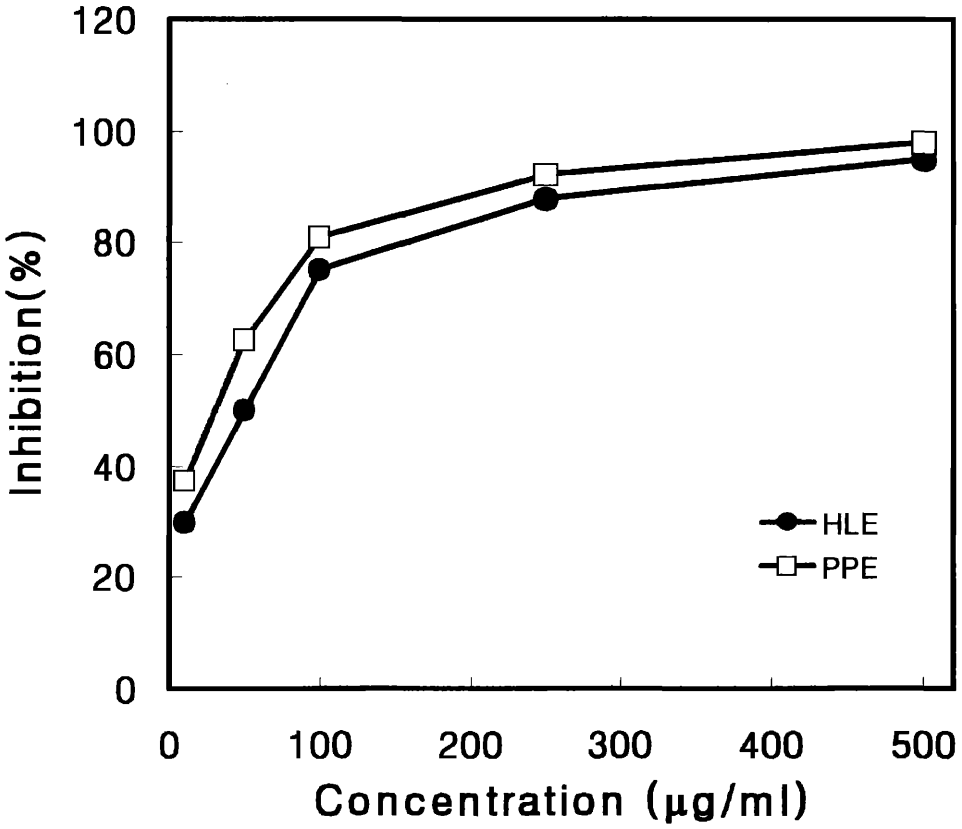

Figure 2. Anti-elastase activity of CC-516. Dose-response curves for the inhibitory effects on PPE (IC_{50}: 40.8 μg/ml) and HLE (IC_{50}: 48.1 μg/ml).

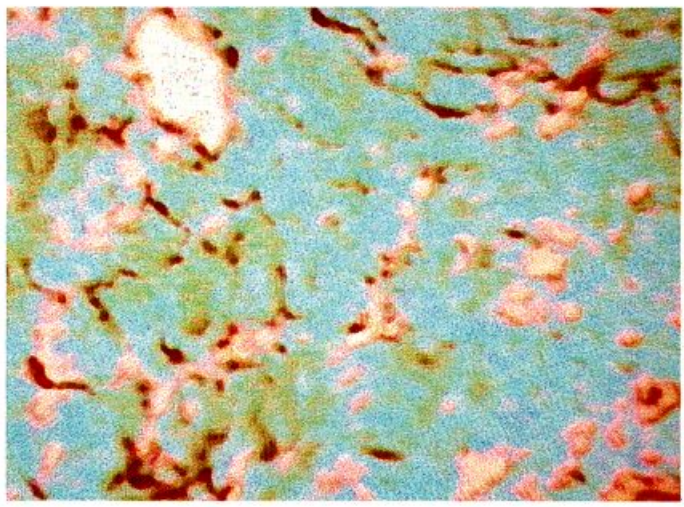

Figure 3. Skin section incubated without elastase. Elastin is the brown-red fibers.

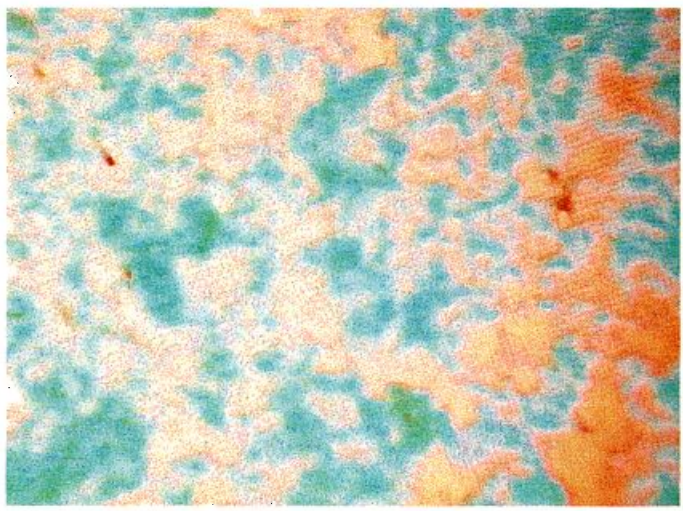

Figure 4. Skin section incubated with 1 μg/ml elastase. Note the total disappearance of elastin fibers.

control, whereas the increase by ascorbic acid was 50%. Data shown in the Table III are the cell viabilities (%) compared to those of the control (100%). The incorporation of amino acids and other compounds in the presence of CC-516 was compared to incorporation alongside ascorbic acid, a known collagen, for synthesis stimulus. Results of the CC-516 are better than those induced by vitamin C when used at its optimal concentration for the synthesis of collagen and the activation of the hydroxylation enzyme (39–42). CC-516 at 10^{-5}% and 10^{-4}% concentration increases the monolayer collagen synthesis by 20% and 50%, respectively. Data shown in Table IV are the synthesis ratio (%) compared to the control (100%). The skin model is a three-dimensional model, close to the living model, which allowed us to confirm the stimulating effect of CC-516 on the synthesis of proteins. At the 0.1% concentration, CC-516 stimulates collagen syn-

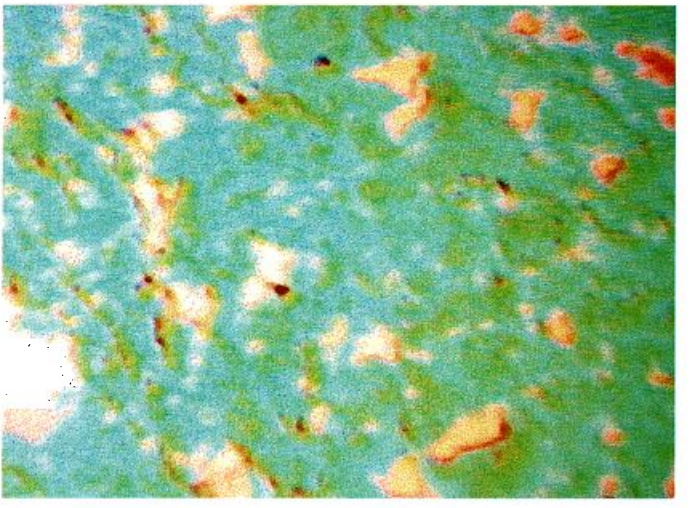

Figure 5. Skin section incubated with elastase (1 μg/ml) in the presence of CC-516 (100 μg/ml).

Table III
Effect of CC-516 on Cell Proliferation at Various Concentrations

	Concentration (%)				
Materials	1×10^{-2}	1×10^{-3}	1×10^{-4}	1×10^{-5}	1×10^{-6}
Ascorbic acid	105	130	150	140	105
CC-516	110	143	185*	120	95
Control	100	100	100	100	100

Data in the table are the cell viabilities (%) compared to control (100%).
* $p < 0.05$ vs control and ascorbic acid.

Table IV
Effect of CC-516 on Collagen Synthesis (monolayer and three-dimensional culture)

Concentration (%)	Monolayer			3-D Culture		
Materials	1×10^{-3}	1×10^{-4}	1×10^{-5}	1×10^{-1}	1×10^{-2}	1×10^{-3}
Ascorbic acid	102	104	118	95	98	92
CC-516	108	140*	120	120	100	104
Control	100	100	100	100	100	100

Data shown in the table are the synthesis ratio (%) compared to control (100%).
* $p < 0.05$ vs control and ascorbic acid.

thesis by a factor of 1.2, at 0.01% and 0.001% concentrations. This stimulation is almost the same as that of the control (100%). The elasticity of the skin depends on its water content and on the swelling capacity of the fibers of its connective tissue. Evaluation of stratum corneum hydration by the capacitance method showed that skin moisturizing increased about 16.5% against untreated cream. In the skin hydration experiment, all measurements were done in triplicate and the average values were used for

statistical evaluation. Both in the cheek region and the eye region, there was no significant difference between the control (base cream) and treated cream (base cream + 3% CC-516) on all the measuring points.

From the results, which are represented in Table V as means of five test persons, it can be concluded that after six weeks of treatment with a test sample, a smaller depth of penetration of the skin into the tube, and thus elasticity, increased by 35%. In the case of the control, no modification was observed. Table V shows the anti-wrinkle effect by three-dimensional image analyzing. The average values were used for statistical evaluation. We applied 2 mg/cm^2 of a control cream or a cream containing 3% of CC-516 twice a day for six weeks to twenty volunteers. The results obtained from the treated side show significant reductions of roughness (−16.7%) and of the average depth of the cutaneous relief (23%), but the untreated skin (control) shows no significant difference. The study of wrinkle depth using SEM reveals apparent reduction in depth of the wrinkles against untreated skin (Figures 6,7), but the control cream showed no significant difference between before and after treatments (not shown).

CONCLUSIONS

The enzyme elastase has received significant attention, primarily because of its reactivity and unspecificity. It is able to attack all major connective tissue matrix proteins, e.g., elastin, collagen, and keratin (43). In contrast to elastase, collagenase is a specific proteinase with a limited number of substrates (44). One major target of cosmetologic research is to find effective elastase inhibitors. Such inhibitors could find their application in anti-wrinkle, anti-aging creams and more generally in numerous compositions intended for skin care. The object of this natural extract is not only to provide an active ingredient that inhibits elastase enzymatic activities (protective role) but also provides the necessary supplies for cells to maintain an appropriate energetic level and to produce their constituents (repairing role). The cells synthesize, secrete, and deposit the proteins of the extracellular matrix made up by collagens, elastin, fibronectin, etc. These proteins ensure the cell inking at the basal plate in the dermal structure. Protecting the skin, its elasticity is the major theme of cosmetic research. The action of a product can be

Table V
Anti-Wrinkle Effect on Roughness and on Mean Depth of Wrinkles and Skin Elasticity of Cream Treated With CC-516

		Anti-wrinkle effect		Elasticity
		Roughness (arbitrary units)	Mean depth (peak depth, μm)	ds (mm)
Untreated cream	Before	32.7	40.5	0.0475
	After	28.3	40.0	0.0480
Treated cream	Before	33.4	52.0	0.0575
	After	28.0	40.0	0.0375

The control is untreated cream before and after six weeks of treatment. Data for skin to which the cream treated with CC-516 has been applied is shown before and after six weeks of treatment. The results obtained on the treated side show significant reduction (−16.7% of roughness and 23% of average depth of wrinkles) compared to before treatment. Skin elasticity increased about 35% compared to before treatment.

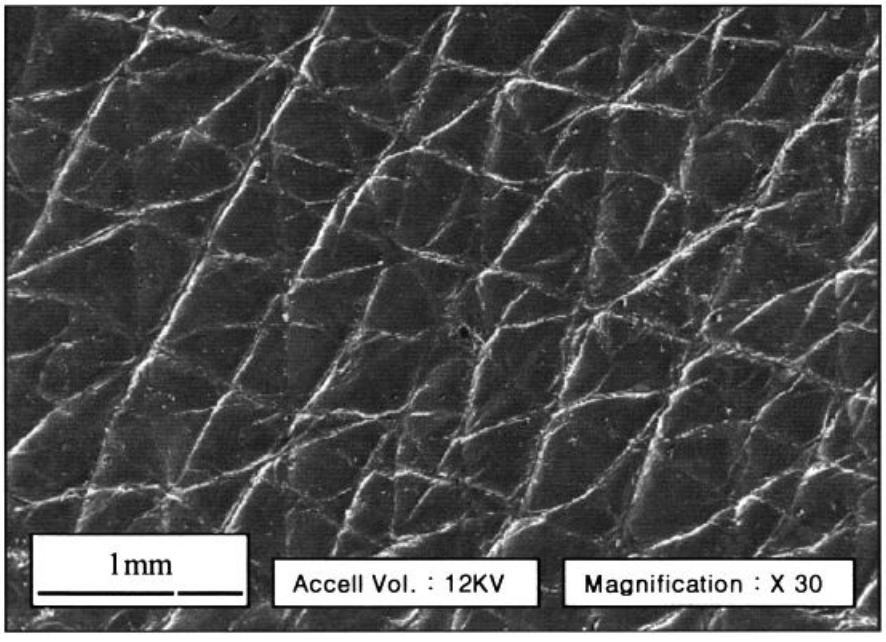

Figure 6. The wrinkles of the skin surface before treatment.

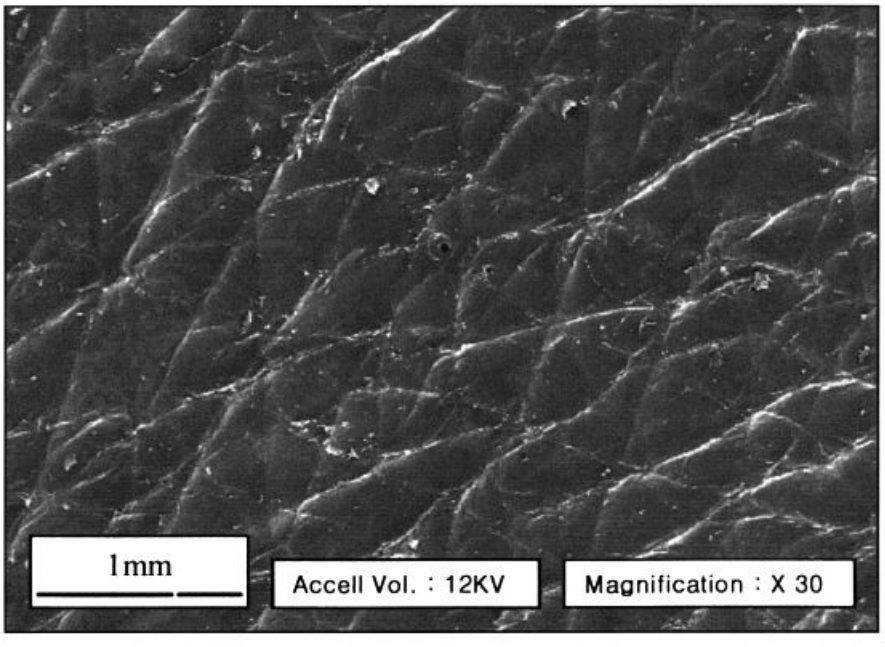

Figure 7. The wrinkles of the skin surface after treatment. The wrinkles decrease in depth after application of the treated cream (3% CC-516).

conceived at several levels. It significantly improves the characteristics of the extracellular matrix, which constitutes the major dermal supporting element. We found enhanced skin elasticity and anti-wrinkle efficacy in a cream containing 3% CC-516. Moreover, its effect on collagen synthesis results in CC-516 being qualified as a restructuring agent, namely by stimulating the collagen synthesis by 120% for 0.1% concentration. This medicinal plant extract is able to preserve collagen and elastin fibers against degradation by hydrolytic enzymes. CC-516 is a new, plant-based, biological elastase inhibitor system and enhances synthesis of the proteins of the extracellular matrix (repairing role). CC-516 can be developed as an anti-aging agent for human skin. In conclusion, we suggest that CC-516 as an active ingredient for anti-aging may be sufficient to satisfy consumers and cosmetic scientists.

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