Anti-Wrinkle Activity of Ziyuglycoside I Isolated from a *Sanguisorba officinalis* Root Extract and Its Application as a Cosmeceutical Ingredient

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In order to investigate the potential of a *Sanguisorba officinalis* root extract as an active ingredient for wrinkle-care cosmetics, we measured its free radical scavenging activity, elastase inhibitory activity, expression of MMP-1 (matrix metalloprotease-1) in vitro, and type I collagen synthesis in normal human fibroblast cells. To isolate the main components from the *S. officinalis* root extract, we purified the extract by solvent fractionation, column chromatography, and recrystallization. The active component was identified as ziyuglycoside I by a spectroscopic analysis. Ziyuglycoside I increased the expression of type I collagen in a dose-dependent manner (by up to 71.3% at 50 μM). A clinical study of a formulation containing ziyuglycoside I, which involved visual evaluation and image analysis, showed a significantly different effect (p < 0.05) of the test formulation from that of the placebo. This result suggests that ziyuglycoside I isolated from *S. officinalis* root extract could be used as an active ingredient for cosmetics.

**Key words:** *Sanguisorba officinalis*; ziyuglycoside I; elastase; matrix metalloproteinase-1 (MMP-1); type I collagen

Elastase is the only enzyme that is capable of degrading elastin, an insoluble elastic fibrous protein in animal connective tissues. It is capable of hydrolyzing nearly all proteins, including supporting and structural proteins of the connective tissue such as collagen and elastin.11 Elastin is the main component of the elastic fibers of the connective tissue and tendons. The elastic fibers in the skin, together with the collagenous fibers, form a network under the epidermis.2 Elastase also plays a critical role in inflammatory processes.31 The enzyme has drawn much attention, primarily because of its reactivity and non-specificity. It is able to attack all major connective tissue matrix proteins, including elastin, collagen, proteoglycans, and keratins. Since this 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 the elastase activity could also be applied as a useful method to protect against skin aging.32 It is generally known that, among the matrix metalloproteinases (MMPs), the enzymes related to collagenase mRNA include MMP-1, MMP-8, MMP-13, and MMP-18.41 MMPs also play critical roles in the degradation of basal membranes and the extracellular matrix for tumor metastasis and permeation in situations such as tumor invasion, migration, and host immune escape, as well as in the destruction of connective tissue due to inflammatory diseases like rheumatoid arthritis, periodontal disease, osteoarthritis, gastric ulcer, and arteriosclerosis from pathological conditions.5,6 MMPs can be divided into four categories according to the preferred substrate: collagenase (MMP-1,8,13,18), gelatinase (MMP-2,9), stromelysin (MMP-3,10), and membrane-type MMPs (MMP-14,15,16,17).7 Since the enzymatic activity of interstitial collagenase to degrade the collagen triple-helix was reported by Gross and Lapiere in 1962,8 a considerable amount of research has been focused on MMP-1 among these matrix metalloproteinases. 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.9–11 The skin consists of two distinct layers, the dermis and epidermis. 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; it also contains proteins, nerves, blood vessels, lymph, and muscles. 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

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direct influence on skin tension. Maintenance of the collagen structure is related to the intrinsic aging and photo-aging processes of the skin.\textsuperscript{12,13} Therefore, a variety of investigations have been focused on protection against skin aging through the inhibition of collagenase activity which disintegrates the ECM proteins.\textsuperscript{14} \textit{Sanguisorba officinalis} is widely distributed throughout Korea and China. Its roots have been used in traditional Oriental medicines for the treatment of burns, scalds, internal hemorrhages, and inflammation.\textsuperscript{15,16} The dried root of \textit{S. officinalis} (Rosaceae), well-known as Ji-Yu in Korea, has been used as a traditional medicine for centuries. It is known to be effective against many types of allergic skin diseases, including urticaria, eczema, and allergic dermatitis.\textsuperscript{17} We have previously screened various plants for free radical scavenging activity and the inhibition of elastase activity, and found that a \textit{S. officinalis} root extract showed a significant inhibitory effect against aging. We investigated the free radical scavenging activities, elastase inhibition activities, reduction of MMP-1 mRNA expression \textit{in vitro}, and type I collagen synthesis in normal human fibroblast cells for the development of potential anti-aging ingredients as raw materials for use in cosmetic products. We isolated the main components from the \textit{S. officinalis} root extract in order to investigate the potential of the \textit{S. officinalis} root extract as an active ingredient in wrinkle-care cosmetics.

\textbf{Materials and Methods}

\textit{Reagents and equipment.} All reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). Other commercially available reagents and solvents were used as received. Human fibroblasts were acquired from ATCC (American Type Culture Collection, CRL-2076). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and the Griess solution were purchased from Sigma (St. Louis, MO, USA) and used as received. Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum, and antibiotics were purchased from Life Technologies (Grand Island, NY, USA). 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\textsuperscript{\textregistered} thermal cycler) were used in the cytotoxicity assay and in the assay on the inhibition of MMP-1 expression. Melting point (mp) data were measured with an Mel-Temp instrument (Laboratory Devices, USA). \textsuperscript{1}H- and \textsuperscript{13}C-NMR spectra were recorded by a Varian-mercury 400 spectrometer. All chemical shifts are reported in parts per million (\delta) relative to tetramethylsilane in a pyridine-\textit{d}_5 solvent.

\textit{Extraction and isolation.} Native plants were collected from all parts of Jeju Island and authenticated by Dr. C. S. Kim, director of the Research Institute for Mt. Halla in Mt. Halla National Park. The collected plants were cut into pieces prior to their use, dried in the shade at room temperature and stored in a dark, cold room until needed. The dried plant material was extracted twice with 70\% (v/v) ethanol (10 times as much as the weight of the dried material) for 24 h at room temperature. The plant extract was passed through filter paper (Whatman, No. 5) and then evaporated at 60\° C, after which the viscous residue was lyophilized to yield the product.

Ziyuglycoside I was isolated from the \textit{S. officinalis} root extract. The air-dried bark of the roots of \textit{S. officinalis} (5 kg) was cut into pieces and extracted twice with EtOH (95\%, 40 kg) for 3 d at room temperature. After passing through a 400-mesh filter cloth, the filtrate was passed through filter paper (Whatman, No. 5) and concentrated under reduced pressure. The combined extract was suspended in H\textsubscript{2}O, and the aqueous suspension was successively extracted with n-hexane, ethyl acetate, and n-BuOH. The ethyl acetate layer was evaporated to dryness to give a residue which was chromatographed on silica gel with a CH\textsubscript{3}Cl\textsubscript{3}-EtOAc-MeOH gradient and divided into 10 fractions. The eighth fraction was rechromatographed on silica gel (CHCl\textsubscript{3}-MeOH, 9:1) and recrystallized from MeOH to yield 50.2 g of white crystalline Ziyuglycoside I (1\% yield, mp 254–258 \° C (dec.). Ziyuglycoside I was identified by \textsuperscript{1}H-NMR and \textsuperscript{13}C-NMR, the results of which were in agreement with previously published data.\textsuperscript{18} Figure 1 shows the structure of ziyuglycoside I.

\textit{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 \textit{et al.}\textsuperscript{18} 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 100, 50, and 10 \mu g/ml, and the samples were then agitated. The optical density at 525 nm was measured after 10 min.

\textbf{Fig. 1.} Structure of Ziyuglycoside I Isolated from the \textit{S. officinalis} Root Extract.
with a UV/Vis spectrophotometer. The free radical scavenging activity of each sample was calculated according to the following formula:

\[
\text{DPPH radical scavenging activity (\%)} = \left[ 1 - \frac{(OD_s - OD_b)}{OD_d} \right] \times 100
\]

where \( OD_s \) is the absorbance of the experimental sample, \( OD_b \) is the absorbance of the blank, and \( OD_d \) 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 activity inhibition. The elastase activity was evaluated according to the method previously reported by Kraunsoe et al.\(^ {10} \) In order to evaluate the inhibition of elastase activity, the amount of released \( p \)-nitroanilide, which was hydrolyzed from the substrate, \( N \)-succinyl-\( \text{Ala-Ala-Ala} \)-\( p \)-nitroanilide, by elastase, was read with a maximum absorbance at 410 nm. In brief, 1.015 mM \( N \)-succinyl-\( \text{Ala-Ala-Ala} \)-\( p \)-nitroanilide was prepared in a 0.1232 M Tris–Cl buffer (pH 8.0), and this solution (1300 \( \mu \)l) was added to each well plate at a density of 100, 50, and 10 \( \mu \)g/ml. The solutions were vortexed and preincubated for 10 min at 25 \( ^\circ \)C, before an elastase (0.0375 unit/ml) stock solution (100 \( \mu \)l) was added. After vortexing, each solution was placed in a water bath for 10 min at 25 \( ^\circ \)C, and the absorbance was measured at 410 nm.

Cell culture. Human normal fibroblast cells were purchased from American Type Culture Collection (ATCC). The cells were cultured in DMEM containing 10\% FBS and 1\% antibiotics at 37 \( ^\circ \)C in 5\% CO\(_2\). The cells were then subcultured with 0.05\% trypsin-0.53 mM EDTA after replacing with 10\% FBS and 1\% antibiotics at 37 \( ^\circ \)C in a humidified atmosphere of 5\% CO\(_2\). After one day, a fresh medium containing 2\% serum was added and the cells were cultured at 37 \( ^\circ \)C in a humidified atmosphere of 5\% CO\(_2\). After one day, a fresh medium containing 2\% serum was added to the cells, which were then treated with a sample for 24 h. Total RNA was isolated from the cells with TRizol (Invitrogen) 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’-TGGGACGAAACA-CATCCTGA-3’ (sense) and 5’-ATCACCTTCTCCCCGATCGT-3’ (anti-sense) for MMP-1; 5’-GAGACCTT-CAACACCAGCAGC-3’ (sense) and 5’-GGCATCTTTGCCTGAAGTTC-3’ (anti-sense) for \( \beta \)-actin. MMP-1 RT-PCR reactions involved reverse transcription at 50 \( ^\circ \)C for 30 min, denaturing at 96 \( ^\circ \)C for 3 min, then 22 cycles of 94 \( ^\circ \)C for 1 min, 48 \( ^\circ \)C for 1 min, and 72 \( ^\circ \)C for 1 min, and finally extension at 72 \( ^\circ \)C for 10 min. The \( \beta \)-actin RT-PCR reactions involved reverse transcription at 50 \( ^\circ \)C for 30 min, denaturing at 96 \( ^\circ \)C for 3 min, then 29 cycles of 94 \( ^\circ \)C for 1 min, 70 \( ^\circ \)C for 1 min, and 72 \( ^\circ \)C for 1 min, and finally extension at 72 \( ^\circ \)C for 10 min. The final products were detected with 1.5\% 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.

Cytotoxicity assay in a monolayer culture. The human fibroblast cells (ATCC, CRL-2076) were seeded in 24-well plates at a density of 1 \( \times \) 10\(^5\) cells per well and cultured at 37 \( ^\circ \)C in 5\% CO\(_2\). After one day, a fresh medium containing 2\% serum was added and the cells were incubated in a CO\(_2\) incubator at 37 \( ^\circ \)C in the presence of a sample for 24 h, before being treated with 100 \( \mu \)l of 2.5 mg/ml of MTT. The cells were then incubated at 37 \( ^\circ \)C for an additional 4 h. The medium containing MTT was discarded, and MTT formazan that had been produced was extracted with 1 ml of DMSO. The absorbance was read at 570 nm with a reference wavelength of 650 nm. The cell viability being calculated as follows:

\[
\text{cell viability (\%)} = \left( \frac{\text{OD}_{570(\text{sample})}}{\text{OD}_{570(\text{control})}} \right) \times 100
\]

where \( \text{OD}_{570(\text{sample})} \) is the absorbance of the treated cells at 570 nm and \( \text{OD}_{570(\text{control})} \) 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 DMEM + 10\% FBS, 50 U/ml of penicillin, and 50 \( \mu \)g/ml of streptomycin at 37 \( ^\circ \)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 100-mm dish at a density of 2 \( \times \) 10\(^6\) cells and cultured at 37 \( ^\circ \)C in 5\% CO\(_2\). After one day, a fresh medium containing 2\% serum was added to the cells, which were then treated with a sample for 24 h. Total RNA was isolated from the cells with TRIzol (Invitrogen) 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’-GGGACGAAACA-CATCCTGA-3’ (sense) and 5’-ATCACCTTCTCCCCGATCGT-3’ (anti-sense) for MMP-1; 5’-GAGACCTT-CAACACCAGCAGC-3’ (sense) and 5’-GGCATCTTTGCCTGAAGTTC-3’ (anti-sense) for \( \beta \)-actin. MMP-1 RT-PCR reactions involved reverse transcription at 50 \( ^\circ \)C for 30 min, denaturing at 96 \( ^\circ \)C for 3 min, then 22 cycles of 94 \( ^\circ \)C for 1 min, 48 \( ^\circ \)C for 1 min, and 72 \( ^\circ \)C for 1 min, and finally extension at 72 \( ^\circ \)C for 10 min. The \( \beta \)-actin RT-PCR reactions involved reverse transcription at 50 \( ^\circ \)C for 30 min, denaturing at 96 \( ^\circ \)C for 3 min, then 29 cycles of 94 \( ^\circ \)C for 1 min, 70 \( ^\circ \)C for 1 min, and 72 \( ^\circ \)C for 1 min, and finally extension at 72 \( ^\circ \)C for 10 min. The final products were detected with 1.5\% 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 collagen type I synthesis by an EIA kit. Fibroblast cells were inoculated into 24-well plates (5 \( \times \) 10\(^5\) cells/well) and cultivated for 24 h. After culturing, the culture medium was changed to serum-free IMDM (Iscove’s modified Dulbecco’s medium) and cultivated for 24 h. The control group was cultivated without a compound. After culturing, the supernatant was collected from each well, and the amount of pro-collagen type I was measured with a pro-collagen type I C-peptide assay kit (Takara Bio, Japan).

In vivo clinical trial. Twenty subjects (35–53-year-old females in good general health) were recruited for this clinical study on a formulation containing ziyuglycoside I. 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 (the test cream contained 0.03\% ziyuglycoside I and the placebo did not). We measured in this
Results and Discussion

Scavenging activity of various plant extracts was measured at each concentration (1–400 μg/ml), the results being shown in Table 1. The scavenging activity (SC_{50} \text{ values for the elastase inhibition activity of \textit{Capsicum annum} (whole plant) and \textit{F. glaberrima} (root), showed more than 50% inhibition at a concentration of 100 μg/ml. Among these, the \textit{S. officinalis} (root) extract was found to have the highest elastase inhibition activity (IC_{50} = 43.0 μg/ml). The IC_{50} values for the elastase inhibition activity of \textit{Capsicum annum} (whole plant) and \textit{F. glaberrima} (root) were 71.1 μg/ml and 88.2 μg/ml, respectively. Notably, \textit{S. officinalis} (root) and \textit{C. annum} (whole plant) showed very high elastase inhibition activity compared to oleanolic acid (IC_{50} = 83.8 μg/ml) which was used as a positive control. The \textit{S. officinalis} root extract also showed significantly high DPPH radical scavenging activity. This result suggested that a \textit{S. officinalis} root extract would have potential as an anti-wrinkle agent for use in cosmetic products.

Table 1. Free Radical Scavenging Activity of Plant Extracts

<table>
<thead>
<tr>
<th>PLANT Botanical name (part used)</th>
<th>DPPH radical scavenging activity (%)</th>
<th>SC_{50}^b (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sanguisorba officinalis (RT)</td>
<td>64.3</td>
<td>7.4</td>
</tr>
<tr>
<td>Camellia japonica (LF)</td>
<td>48.7</td>
<td>7.4</td>
</tr>
<tr>
<td>Capsicum annuum (WP)</td>
<td>21.3</td>
<td>345.3</td>
</tr>
<tr>
<td>Cassia nomame (WP)</td>
<td>91.2</td>
<td>20.7</td>
</tr>
<tr>
<td>Crinum asiaticum (RT)</td>
<td>0.8</td>
<td>&gt;400</td>
</tr>
<tr>
<td>Daphniphyllum macropodum (LF)</td>
<td>81.4</td>
<td>62.1</td>
</tr>
<tr>
<td>Filipendula glaberrima (RT)</td>
<td>88.7</td>
<td>20.5</td>
</tr>
<tr>
<td>Pyrosyra hastata (RT)</td>
<td>83.9</td>
<td>30.1</td>
</tr>
<tr>
<td>Solanum tuberosum L. (RH)</td>
<td>32.5</td>
<td>25.4</td>
</tr>
<tr>
<td>BHT</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

^aParts used were LF (leaf), RT (root), WP (whole plant) and RH (rhizome); BHT (di-β-butyl hydroxy toluene).

^bSC_{50} indicates the concentration (μg/ml) at which the percentage inhibition of the DPPH radical scavenging activity was 50%.

Free radical scavenging activity

It has been reported that free radicals induced by ultraviolet light or oxidative stress accelerate skin aging. Assays of the free radical scavenging capacity were carried out by the DPPH method. The free radical scavenging capacity of various native plant extracts was measured at each concentration (1–400 μg/ml), the results being shown in Table 1. The free radical scavenging capacity is expressed as SC_{50}, the concentration needed to reduce 50% of the DPPH radical. Four extracts, \textit{Sanguisorba officinalis} (root), \textit{Cassia nomame} (whole plant), \textit{Filipendula glaberrima} (root), and \textit{Pyrosyra hastata} (root) showed efficient free radical scavenging capacity (SC_{50} < 30 μg/ml). Among these, the \textit{S. officinalis} (root) extract had the highest free radical scavenging activity (SC_{50} = 7.4 μg/ml). The SC_{50} values for \textit{C. nomame} (whole plant) and \textit{F. glaberrima} (root) were 20.7 μg/ml and 20.5 μg/ml, respectively. These extracts showed very high free radical scavenging activity compared to BHT (di-β-butyl hydroxytoluene; SC_{50} = 25.4 μg/ml) which was used as a positive control.

Inhibition of the elastase activity

We had previously screened various plant extracts for their elastase inhibition activities. Table 2 showing the results of the preliminary screening test. Three plant extracts, \textit{S. officinalis} (root), \textit{Capsicum annum} (whole plant), and \textit{Filipendula glaberrima} (root), showed more than 50% inhibition at a concentration of 100 μg/ml. Among these, the \textit{S. officinalis} (root) extract was found to have the highest elastase inhibition activity (IC_{50} = 43.0 μg/ml). The IC_{50} values for the elastase inhibition activity of \textit{Capsicum annum} (whole plant) and \textit{F. glaberrima} (root) were 71.1 μg/ml and 88.2 μg/ml, respectively. Notably, \textit{S. officinalis} (root) and \textit{C. annum} (whole plant) showed very high elastase inhibition activity compared to oleanolic acid (IC_{50} = 83.8 μg/ml) which was used as a positive control. The \textit{S. officinalis} root extract also showed significantly high DPPH radical scavenging activity. This result suggested that a \textit{S. officinalis} root extract would have potential as an anti-wrinkle agent for use in cosmetic products.

Cytotoxicity assay in a monolayer culture

Triterpenes have drawn strong attention due to their pharmacological activities, for example, oleanolic acid and ursolic acid are triterpenoid compounds that exist widely in food, medicinal herb and other plants. These triterpenes have been well studied for their pharmacology-like anti-inflammatory and antioxidative effects in previously reported work. Furthermore, these triterpenes are now used by the cosmetic industry on the assumption that they have a regenerative or anti-aging effect. The constituents of the \textit{S. officinalis} root have been studied since Yosioka \textit{et al.} have previously reported on ziyuglycoside I, ziyuglycoside II and po-
The results of HPLC assay show ziyuglycoside I to be the richest constituent among the triterpene glycosides and their aglycones. Ziyuglycoside I did not show cytotoxicity compared with other triterpenes such as ziyuglycoside II and tomentosolic acid (data not shown). We therefore focused on ziyuglycoside I and its potential as a cosmetic ingredient.

In order to evaluate the cytotoxicity of the *S. officinalis* root extract and ziyuglycoside I 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 Figs. 2 and 3 at a concentration of 100 μg/ml, the *S. officinalis* root extract showing negligible cytotoxicity compared to that of the positive control. Ziyuglycoside I showed no cytotoxicity up to the effective concentration for anti-wrinkle activity (less than 50 μM). These findings suggest that Ziyuglycoside I could be produced as an effective active ingredient with no associated cytotoxicity.

**Table 2. Elastase Inhibition Activity of Plant Extracts**

<table>
<thead>
<tr>
<th>PLANT Botanical name (part used)</th>
<th>Inhibition (%)</th>
<th>IC₅₀ (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>100μg/ml</td>
<td>50μg/ml</td>
</tr>
<tr>
<td><em>Sanguisorba officinalis</em> (RT)</td>
<td>65.3</td>
<td>56.2</td>
</tr>
<tr>
<td><em>Camellia japonica</em> (LF)</td>
<td>45.2</td>
<td>33.1</td>
</tr>
<tr>
<td><em>Capsicum annum</em> (WP)</td>
<td>61.7</td>
<td>41.4</td>
</tr>
<tr>
<td><em>Cassia nomame</em> (WP)</td>
<td>29.9</td>
<td>22.8</td>
</tr>
<tr>
<td><em>Crinum asiaticum</em> (RT)</td>
<td>14.2</td>
<td>16.3</td>
</tr>
<tr>
<td><em>Daphniphyllum macropodum</em> (LF)</td>
<td>—</td>
<td>5.9</td>
</tr>
<tr>
<td><em>Filipendula glaberrima</em> (RT)</td>
<td>52.8</td>
<td>40.8</td>
</tr>
<tr>
<td><em>Pyrossia hastata</em> (RT)</td>
<td>48.5</td>
<td>31.5</td>
</tr>
<tr>
<td><em>Solanum tuberosum</em> L. (RH)</td>
<td>40.4</td>
<td>38.0</td>
</tr>
<tr>
<td>Oleanolic acid</td>
<td>57.7</td>
<td>35.2</td>
</tr>
</tbody>
</table>

aParts used were LF (leaf), RT (root), WP (whole plant) and RH (rhizome).

bIC₅₀ indicates the concentration (μg/ml) at which the percentage inhibition of elastase activity was 50%.

In order to screen anti-aging candidates that may inhibit the degradation of collagen fibers, we investigated the reduction of MMP-1 expression by seven extracts with superior elastase inhibition activity 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 and to have a protective effect against skin damage caused by UV rays. The MMP-1 expression assay on human fibroblasts was carried out with a Gel Doc 2000 image analyzer (Bio-Rad). The *S. officinalis* root extract reduced the expression of MMP-1 (by up to 40.6% at 50 μg/ml) as shown in Fig. 4. The *S. officinalis* root extract at a final concentration of 25–50 μg/ml exhibited 6.8–40.6% inhibition of MMP-1 expression.
Assay of collagen type I synthesis by an EIA kit

To evaluate the amount of collagen type I synthesis that occurred upon exposure to the extract, collagen type I was quantitatively detected by using a pro-collagen type I C-peptide assay kit (Takara Bio, Japan). Collagens are synthesized as precursor molecules, called procollagens. These molecules contain additional peptide sequences, usually referred to as “propeptides,” at both the amino-terminal end and the carboxy-terminal end. These propeptides are cleaved from the collagen triple-helix molecule during its secretion, after which the triple-helix collagens are polymerized into extracellular collagen fibrils. Thus, the amount of free propeptide stoichiometrically reflects the amount of collagen molecules synthesized. Ziyuglycoside I isolated from the S. officinalis root extract increased the expression of type I collagen in a dose-dependent manner (up to 71.3% at 50 μM), comparable to that of ascorbic acid (up to 75.2% at 250 μM) as shown in Fig. 5. Ziyuglycoside I in a concentration range from 10–50 μM showed an increase in type I collagen synthesis from 30.4% to 71.3%.

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, 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. The results show that the difference between the test group and the placebo group was not significant until 4 and 8 weeks after the treatment, but that there was a significant difference 12 weeks after the treatment (Fig. 6). In an image analysis of skin replicas by using 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, 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 showed greatest improvement of the average difference in roughness (ΔR3, −0.03 ± 0.02) than the placebo formulation (ΔR3; −0.01 ± 0.02) (Fig. 7). 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 (Fig. 8). These results therefore suggest that the test substance could be described as an aid in anti-wrinkling.

In order to investigate the potential of various plant extracts as active ingredients for wrinkle-care cosmetics, we screened their free radical scavenging activities and elastase inhibition activities. S. officinalis (root), C. no-
mame (whole plant), and F. glaberrima (root) showed higher free radical scavenging activities than BHT (di-t-butyl hydroxytoluene), a synthetic antioxidant. Among these, the S. officinalis root extract showed the highest free radical scavenging activity. S. officinalis (root) and C. annum (whole plant) showed very high elastase inhibition activity compared to that of oleanolic acid which was used as a positive control. The S. officinalis root extract was found to have the highest elastase inhibition activity, and also showed high DPPH radical scavenging activity. When comparing the inhibitory activity in terms of the MMP-1 expression in human fibroblasts, with EGCG used as a positive control, the S. officinalis root extract reduced the expression of MMP-1 in a dose-dependent manner.

To isolate the main components of the S. officinalis root extract, we purified the extract through solvent fractionation, column chromatography, and recrystallization. Among these compounds, ziyuglycoside I was identified from spectroscopic evidence. Ziyuglycoside I isolated from the S. officinalis root extract did not show comparable DPPH radical scavenging activity (36% at 3 mg/ml) to that of the S. officinalis root extract (SC$_{50}$ = 7.4 µg/ml). The inhibitory activities of ziyuglycoside I toward elastase and MMP-1 (5.4% at 667 µg/ml and 6.2% at 100 µg/ml) were also negligible compared to...
those of the S. officinalis root extract (IC50 = 43.0 μg/mL). However, ziyuglycoside I strongly increased the expression of type I collagen in a dose-dependent manner comparable to that of ascorbic acid which was used as a positive control. The S. officinalis root extract showed negligible cytotoxicity at a concentration of 100 μg/mL and ziyuglycoside I did not show any cytotoxicity up to the effective concentration for anti-wrinkle activity. The visual evaluation and image analysis used in this clinical study showed a statistically significant difference (p < 0.05) between the effects of the tested formulation and the placebo. All of these results suggest that ziyuglycoside I isolated from the S. officinalis root extract could be used as an active ingredient in new anti-wrinkle cosmetic products.

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References


