

온침음 물 추출물의 세포독성, 피부재생, 주름개선, 미백 및 보습 효과

대구한의대학교 한의과대학 부인과학교실
안뜰에봄, 김동철

ABSTRACT

In Vitro Cytotoxicity, Skin Regeneration, Anti-wrinkle, Whitening and *In Vivo* Skin Moisturizing Effects of *Oncheongeum*

Tteul-E-Bom An, Dong-Chul Kim

Dept. of Obstetrics & Gynecology, College of Korean Medicine,
Daegu Haany University

Objectives: The objective of this study was to evaluate the effects of cytotoxicity, skin regeneration, anti-wrinkle, whitening and skin moisturizing of *Oncheongeum* (OCE).

Methods: The cytotoxicity of OCE lyophilized aqueous extracts (yield = 13.82%) was observed against human normal fibroblast cells and B16/F10 murine melanoma cells by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium Bromide (MTT) assay, and skin regeneration and anti-wrinkle effects were also evaluated through the assay of collagen type I synthesis compared to the transformation of the growth factor (TGF)- β 1, hyaluronidase, collagenase and matrix metalloproteinase (MMP)-1 inhibitory assays compared to oleanolic acid (OA), and elastase inhibitory effects compared to phosphoramidon disodium salt (PP). In addition, OCE's whitening effects were measured by a tyrosinase inhibitory assay and melanin formation test in B16/F10 murine melanoma cells compared to arbutin, and skin moisturizing effects were observed through a mouse skin water content test, respectively.

Results: No OCE treatment-related cytotoxic effects appeared on human normal fibroblasts and B16/F10 murine melanoma cells. OCE concentration-dependently increased the collagen Type I synthesis on human normal fibroblast cells, and also effectively inhibited hyaluronidase, elastase, collagenase and MMP-1 activities. In addition, OCE inhibited melanin production of B16/F10 murine melanoma cells and activity of tyrosinase. And significant and dose-dependent increases of skin water content were detected in OCE-treated mouse skin compared to vehicle control skins.

Conclusions: OCE showed favorable and sufficient effects in skin regeneration, anti-wrinkle, whitening and skin moisturizing in this experiment. But more detail mechanisms and studies on the skin protective efficiency of *in vivo* are needed with the screening of active biological compounds in individual OCE herbs.

Key Words: *Oncheongeum*, skin regeneration, anti-wrinkle, whitening, moisturizing

I. Introduction

The phenomenon of skin aging is related to various cell structures and extracellular components and express clinical signs including irregular dryness, dark/light pigmentation, sallowness, deep furrows or severe atrophy, dehydrogenation, telangiectases, premalignant lesions, laxity and a leathery appearance¹⁾. For women, skin aging can not only mean older age but also loss of physical attraction, so interest in skin care is rising in gynecology²⁾.

《Somun · Sanggocheonjinnon》 said that for women, face color and gloss change occur over a seven-year period after age 28³⁾.

The female face turns sallow due to a sudden change in the body including decrease in metabolic ability, waste accumulation and erosion of self-purification capacity brought about by delivery, and women experience menopause with the accompanying symptoms of facial blushing due to a female hormone imbalance in their 40s. In addition, their health declines because of stress and fatigue caused by doing chores and engaging in social activities, and thus their skin also undergoes change. Pigment around the face increases because of accumulated ultraviolet irradiation⁴⁾. So the Korean medical sector must study development of new ingredients to improve skin health by inhibiting the skin aging process

brought about by environmental and physiological factors.

Oncheongeum aqueous extract (OCE), also called “*Haedoksamultang*”, was mainly used for healing metrorrhagia. Now, OCE is used for treating a broad spectrum of diseases⁵⁾. And various treatment effect of OCE have been suggested through experiments, showing few or no obvious side effects at clinics⁶⁻⁹⁾. But the study of anti-aging suffers from a lack of research on things like skin regeneration and whitening. The expectation, however, is that OCE has also show favorable effects in skin regeneration, anti-wrinkle, whitening and skin moisturizing through known and potent anti-oxidative effects on skin aging¹⁰⁾. So we intend to observe the effects of cytotoxicity, skin regeneration, anti-wrinkle, whitening and skin moisturizing from lyophilized powder of OCE in this study.

II. Materials and methods

1. Preparation and administration of test materials

Eight types of herbs consisted of OCE were purchased from the herbal store (Jecheonhanbangyakcho, Jecheon, Korea), and the composition of individual herbs and product regions were listed in Table 1. A combined 23 g of the appropriate portions of individual herbs consisted of OCE were boiled in 1,000 ml of distilled water for four hours three times at 60°C,

and evaporated using an automated round flask evaporator (Eyela N-1110, Tokyo, Japan), then completely lyophilized using a programmable freeze dryer. Another 3.18 g of lyophilized OCE aqueous extract were acquired and stored at minus 20°C in a refrigerator to protect from light and humidity until used. Colorless to white powder of arbutin, white powder of TGF-β1, white to slightly yellow

solid of Phosphoramidon disodium salt (PP) and white solid of Oleanolic acid (OA) were used as standard reference material for the effects of skin regeneration, anti-wrinkle and whitening, respectively. The powders were also stored at minus 20°C in a refrigerator to protect from light and humidity until used in this study.

Table 1. Composition of OCE Used in this Study

Herbs	Scientific name	Korean name	Produce region	Amounts (g)
<i>Angelicae Gigantis Radix</i>	<i>Angelica gigas</i> Nakai	當歸	Pyeongchang, Gangwon	4.00
<i>Rehmanniae Radix</i>	<i>Rehmannia glutinosa</i> (Gaertner) Liboschitz ex Steudel	乾地黄	Jecheon, Chungbuk	4.00
<i>Paeoniae Radix</i>	<i>Paeonia lactiflora</i> Pallas	白芍藥	Jecheon, Chungbuk	3.00
<i>Cnidii Rhizoma</i>	<i>Cnidium officinale</i> Makino	川芎	Youngyang, Gyeongbuk	3.00
<i>Scutellariae Radix</i>	<i>Scutellaria baicalensis</i> Georgi	黃芩	Jeongseon, Gangwon	3.00
<i>Coptidis Rhizoma</i>	<i>Coptis japonica</i> Makino	黃蓮	China	2.00
<i>Phellodendri Cortex</i>	<i>Phellodendron amurense</i> Ruprecht	黃柏	Yeongwol, Gangwon	2.00
<i>Gardeniae Fructus</i>	<i>Gardenia jasminoides</i> Ellis	梔子	Jindo, Chunnam	2.00
Total	8 types			23.00

2. Cytotoxicity assay in a monolayer culture

Human normal fibroblast cells (CRL-2076; ATCC, Manassas, VA, USA) and B16/F10 murine melanoma cells (CRL-6475; ATCC, Manassas, VA, USA) were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich, St. Louise, MO, USA) containing 10% fetal bovine serum (Gibco BRL, Grand Island, NY, USA), 100 units/ml of penicillin (Sigma-Aldrich, St. Louise, MO, USA) and 100

µg/ml of streptomycin (Sigma-Aldrich, St. Louise, MO, USA) at 37°C in a humidified atmosphere of 5% CO₂. Then the cells were subcultured with 0.05% trypsin-0.53 mM EDTA (Sigma-Aldrich, St. Louise, MO, USA) after replacing with a fresh medium every two or three days. In B16/F10 murine melanoma cells, 2 mL-glutamine (Sigma-Aldrich, St. Louise, MO, USA) were additionally supplied into cultured medium. The human fibroblast cells and B16/F10

murine melanoma cells were seeded in 24-well plates at a density of 1×10^5 cells/well and cultured at 37°C in 5% CO₂. After one day, a fresh medium containing 2% serum was added and the cells were incubated in a CO₂ incubator at 37°C in the presence of samples (1.25, 2.5, 5, 10, 100 and 500 mg/ml of OCE) for 24 hrs, before being treated with 100 µl of 2.5 mg/ml of MTT (Sigma-Aldrich, St.Louise, MO, USA). The cells then incubated at 37°C for an additional 4 hrs. The medium containing MTT was discarded, and MTT formazan that had been produced was extracted with 1 ml of Dimethyl sulfoxide (DMSO). The absorbance was read at 570 nm with a reference wavelength of 650 nm with a microplate reader (Tecan, Männedorf, Switzerland). The cell viability was calculated as follow equation[1].

$$\text{Equation[1]. Cell viability (\%)} = \frac{\text{OD570 (sample)}}{\text{OD570 (control)}} \times 100$$

Where OD570 (sample) is the absorbance of the treated cells at 570 nm and OD570 (control) is the absorbance of the negative control at 570 nm (non-treated cells).

3. Skin regeneration and anti-wrinkle effects

The skin regeneration and anti-wrinkle effects of test samples were also observed through the collagen type I synthesis assay¹¹⁾, the inhibition of hyaluronidase¹²⁾, elastase¹³⁾, collagenase¹⁴⁾ and MMP-1¹⁵⁾

in this study.

1) Assay of collagen type I synthesis by an EIA kit

The anti-wrinkle effects of test samples were observed through the collagen type I synthesis assay¹¹⁾ in this experiment. Briefly, fibroblast cells were inoculated into 24-well plates (5×10^5 cells/well) and cultivated for 24 hrs. After culturing, the culture medium was changed to serum-free Iscove's modified Dulbecco's medium (IMDM; Sigma-Aldrich, St. Louise, MO, USA) and cultivated for 24 hrs containing 0.0125, 0.025, 0.05, 0.1, 1 and 10 mg/ml of test samples (OCE) or 0.1, 1, 10, 20, 40 and 100 ng/ml of TGF-β1. 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 (TakaraBio, Tokyo, Japan), read at 450 nm with a microplate reader. The pro-collagen synthesis was calculated as follow equation[2] and the results were reported in terms of EC₅₀.

$$\text{Equation[2]. Pro-collagen synthesis (\%)} = \frac{\text{OD450 (sample)}}{\text{OD450 (control)}} \times 100$$

2) Hyaluronidase inhibitory assay

The assay was performed according to the method reported earlier¹²⁾. Hyaluronidase reacts with the substrate hyaluronic acid to release N-acetyl glucosamine. In presence of any inhibitors, the release of N-acetyl glucosamine is reduced and it is monitored by measuring the absorbance

at 600 nm. The inhibitory activity of the OCE (0.0125, 0.025, 0.05, 0.1, 1 and 10 mg/ml) was compared with OA used as a standard reference material under exactly the same experimental conditions. The OD at 600 nm was measured after 15 min with a 96 well microplate reader (Tecan, Männedorf, Switzerland) and hyaluronidase inhibitory activity of each sample was calculated according to the following equation[3]. The results were reported in terms of IC₅₀.

Equation[3]. Hyaluronidase inhibitory activity (%) = 100 - [(OD600 (sample) + OD600 (control)) / OD600 (control)] × 100

3) Elastase inhibitory assay

The elastase inhibition assay was performed by measuring the release of p-nitro aniline due to proteolysis of N-succinyl-(Ala)₃-p-nitroanilide by human leucocyte elastase (Sigma-Aldrich, St. Louise, MO, USA)¹³⁾ in the presence or absence of the OCE (12.5, 25, 50, 100, 200 and 400 mg/ml) or PP (0.625, 1.25, 2.5, 5, 10 and 100 ng/ml) as a standard under exactly the same experimental conditions. The absorbance was measured at 410 nm with a 96 well microplate reader and elastase inhibitory activity of each sample was calculated according to the following equation[4]. The results were reported in terms of IC₅₀.

Equation[4]. Elastase inhibitory activity (%) = 100 - [(OD410 (sample) / OD410 (control)) × 100]

4) Collagenase inhibitory assay

The collagenase inhibition assay was

performed according to the method that has been reported previously by Niemann¹⁴⁾. 0.15 ml of collagenase (1 mg/ml; Sigma-Aldrich, St. Louise, MO, USA) was added to the mixed solutions consisted of 2 mM 4-phenylazobenzyloxycarbonyl-pro-leu-gly-pro-d-ar (Sigma-Aldrich, St. Louise, MO, USA) and 0.25 ml and 0.1 ml of OCE (0.0125, 0.025, 0.05, 0.1, 1 and 10 mg/ml) in 0.1 M Tris-HCl buffer (pH 7.5) and then reacted for 20 mins at 37°C. After that, the reactions were stopped by adding 6% citric acid (Daejung, Seoul, Korea) 0.5 ml. Absorbance was measured at 320 nm with a UV/Vis spectrophotometer after addition of ethyl acetate (Sigma-Aldrich, St. Louise, MO, USA) 1.5 ml and collagenase inhibitory activity of each sample was calculated according to the following equation[5]. The results were reported in terms of IC₅₀. OA (0.0125, 0.025, 0.05, 0.1, 1 and 10 mg/ml) was used as a standard under exactly the same experimental condition.

Equation[5]. Collagenase inhibitory activity (%) = 100 - [(OD320 (sample) / OD320 (control)) × 100]

5) MMP-1 inhibitory assay

The assay was performed over fluorescence microplate according to the previous report¹⁵⁾ with slight modification. Briefly, 20 µl of type-I collagen (substrate; Sigma-Aldrich, St. Louise, MO, USA) was mixed with 80 µl of each of diluted OCE or OA (0.0125, 0.025, 0.05, 0.1, 1 and 10 mg/ml) as a standard under exactly

the same experimental conditions. Then 100 μ l of diluted (0.2 U/ml) MMP-1 (Sigma-Aldrich, St. Louise, MO, USA) was added to each well and the plate was incubated at room temperature for 1-2 hrs protected from light. Fluorescence was measured at excitation maxima at 495 nm and emission maxima at 515 nm. All the dilutions were made with reaction buffer containing 0.5 M Tris-HCl, 1.5 M NaCl, 50 mM CaCl₂ and 2 mM sodium azide at pH 7.6. The control used for this experiment was the buffer with the substrate and the inhibitors without MMP-1. MMP-1 inhibitory activity of each sample was calculated according to the following equation[6]. The results were reported in terms of IC₅₀.

Equation[6]. MMP-1 inhibitory activity (%) = $100 - [(OD_{515}(\text{sample}) / OD_{515}(\text{control})) \times 100]$

4. Whitening effects

The whitening effects of OCE were measured by tyrosinase inhibitory assay¹⁶⁾ and melanin formation test in B16/F10 murine melanoma cells¹⁷⁾ in this study.

1) Tyrosinase inhibitory assay

Tyrosinase inhibition was assayed according to the method of Masamoto¹⁶⁾. Briefly, aliquots (0.05 ml) of OCE (12.5, 25, 50, 100, 200 and 400 μ g/ml) were mixed with 0.5 ml of L-DOPA (Sigma-Aldrich, St. Louise, MO, USA) solution (1.25 mM), 0.9 ml of sodium acetate buffer solution (0.05 M, pH 6.8) and preincubated at 25°C for 10 mins. Then,

0.05 ml of an aqueous solution of mushroom tyrosinase (333 U/ml; Sigma-Aldrich, St. Louise, MO, USA) was added last to the mixture. This solution was immediately monitored for the formation of dopachrome by measuring the linear increase in OD at 475 nm with a UV/Vis spectrophotometer and tyrosinase inhibitory activity of each sample was calculated according to the following equation[7]. The results were reported in terms of IC₅₀. Arbutin (10, 20, 40, 80, 160 and 320 μ g/ml) was used as a standard under exactly the same experimental condition.

Equation[7]. Tyrosinase inhibitory activity (%) = $100 - [(OD_{475}(\text{sample}) / OD_{475}(\text{control})) \times 100]$

2) Melanin formation test in B16/F10 murine melanoma cells

B16/F10 murine melanoma cells were harvested by trypsinization when they were about 70 percent confluent, counted with a haemocytometer and seeded at the appropriate numbers into wells of cell culture plates for further experiments. The melanin content was measured by the way it adds a little modification to be described in the previous investigation¹⁷⁾.

The B16/F10 murine melanoma cells were seeded with 2×10^5 cells/well in three ml of medium in six-well culture plates and incubated overnight to allow cells to adhere. The cells were exposed to various concentrations of OCE (50, 100, 200, 400, 800 and 1,600 μ g/ml) for 72 hours in the presence or absence of

100 nM alpha-melanocyte stimulating hormones (α -MSH, Sigma-Aldrich, St. Louise, MO, USA) At the end of the treatment, the cells were washed with PBS and lyzed with 800 μ l of 1 N NaOH (Merck, Darmstadt, Germany) containing 10 percent DMSO (Sigma-Aldrich, St. Louise, MO, USA) for an hour at 80°C. Absorbance at 400 nm was measured using a microplate reader. The melanogenesis inhibitory activity of each sample was calculated according to the following equation[8]. The results were reported in terms of IC₅₀. Arbutin (20, 40, 80, 160, 320 and 640 μ g/ml) was used as a standard under the same experimental condition.

Equation[8]. Melanin produces inhibitory activity (%) = $100 - [(OD_{400}(\text{sample}) / OD_{400}(\text{control})) \times 100]$.

5. *In vivo* skin moisturizing effects

The skin moisturizing effects of test materials were measured by the changes of mouse skin water contents test^{18,19)} in this study.

1) Animal and husbandry

Forty-one male SPF/VAF Outbred CrljOri:CD1[ICR] mice (6-wks old upon receipt, OrientBio, Seungnam, Korea: ANNEX I and II) were used in this study after 7 days of acclimatization. Animals were allocated four to five per polycarbonate cage in a temperature (20~25°C) and humidity (40~45%) controlled room. Light:dark cycle was 12 hrs: 12 hrs and normal rodent pellet

diet and water were supplied free to access during acclimatization. After acclimatization, eight mice in each group were selected based on the body weights (Mean 31.53 \pm 2.14 g, ranged in 27.50~35.20 g), and divided into four groups: vehicle control, OCE 500, 250 and 125 mg/kg orally administered groups. All laboratory animals were treated according to the national regulations of the usage and welfare of laboratory animals, and approved by the Institutional Animal Care and Use Committee in Daegu Haany University (Gyeongsan, Korea) prior to animal experiment [Approval No DHU2015-030, 2015. 04. 13; ANNEX III]. All animals were overnight fasted for 18 hrs before initial administration and sacrifice (water was not restricted) to reduce individual differences from feeding in this study.

2) Treatment

OCE were dissolved in distilled water as 50, 25 and 12.5 mg/ml concentrations and orally administered in a volume of 10 ml/kg as equivalence to 500, 250 and 125 mg/kg, once a day for 7 days by gastric gavages using a zoned attached to 1 ml syringe. In vehicle control, only distilled water was orally administered instead of OCE solutions in this study.

3) Body weight measurement

Changes of body weight were measured at 1 day before initial test article administration, the day of first test material administration, 1, 3, 6 and 7 days after initial vehicle or OCE administration

using an automatic electronic balance (Precisa Instrument, Zuerich, Switzerland). To reduce the individual differences, the body weight gains during 7 days of experimental periods were calculated as follow equation[9] according to previous report²⁰⁾.

Equation[9]. Body Weight Gains (g) = (Body weight at 24 hrs after last 7th test substance administration - body weight at the day of initial administration)

4) Skin water content measurement

At 24 hrs after end of last 7th administration of vehicle or OCE solutions, 2×3 cm of hair clipped dorsal skin samples were removed, and skin water contents (%) were measured by automated moisture analyzers balance (MB23, Ohaus, Pine Brook, NJ, USA), according to previous report¹⁸⁾. In addition, the percent-point changes compared to that of vehicle control were calculated to help the understanding of the efficacy of test materials as follow equation[10] according to previous report²⁰⁾.

Equation[10]. Percent-point changes compared with vehicle control (%) = $[(\text{Data of OCE administration groups} - \text{Data of vehicle treated control}) / \text{Data of vehicle treated control}] \times 100$

6. Statistical analyses

All *in vitro* data were expressed as mean±SD of five independent experiments, and skin water contents were calculated as mean±SD of eight mice skins at each time points. Multiple comparison

tests for different dose groups were conducted. Variance homogeneity was examined using the Levene test²¹⁾. If the Levene test indicated no significant deviations from variance homogeneity, the obtain data were analyzed by one way ANOVA test followed by least-significant differences multi-comparison (LSD) test to determine which pairs of group comparison were significantly different. In case of significant deviations from variance homogeneity was observed at Levene test, a non-parametric comparison test, Kruskal-Wallis H test was conducted. When a significant difference is observed in the Kruskal-Wallis H test, the Mann-Whitney U (MW) test was conducted to determine the specific pairs of group comparison, which are significantly different²²⁾. Differences were considered significance at P<0.05. EC₅₀ or IC₅₀ values in each *in vitro* assay were calculated by Probit methods and statistical analyses were conducted using SPSS for Windows (Release 14.0K, SPSS Inc., Chicago, IL, USA)¹⁸⁾.

III. Results

1. Cytotoxic effects

1) Against human normal fibroblast cells
OCE treatment didn't have significantly cytotoxic effects and the changes on the human normal fibroblast cell viabilities, from 1.25 to 500 mg/ml concentrations, the lowest to the highest concentrations tested in this study (Fig. 1).

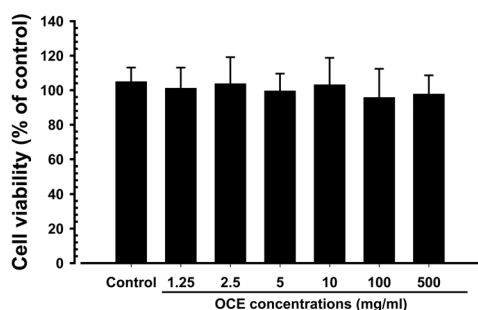


Fig. 1. Effects of OCE on the human normal fibroblast cell viabilities.

2) Against B16/F10 murine melanoma cells

Any significant changes on the B16/F10 murine melanoma cell viabilities were not observed in all six concentrations (from 1.25 to 500 mg/ml) of OCE treated cells as compared with non-treated vehicle control, suggesting no cytotoxicity of test samples against B16/F10 murine melanoma cells in this study (Fig. 2).

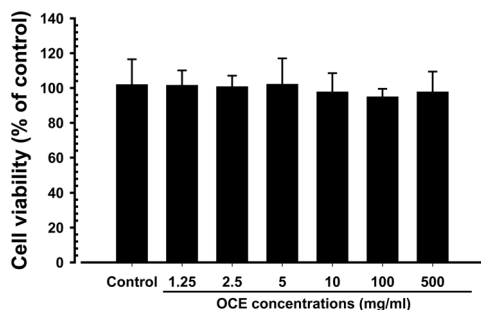


Fig. 2. Effects of OCE on the B16/F10 murine melanoma cell viabilities.

2. Skin regeneration and anti-wrinkle effects

1) Effect on the collagen type I synthesis

Significant ($p < 0.01$) increases of the type I collagen synthesis were detected from 10 ng/ml of TGF- β 1 and 0.05

mg/ml concentration of OCE treated fibroblasts, respectively. And EC_{50} of TGF- β 1 and OCE on the type I collagen synthesis were detected as 18.59 ± 6.19 ng/ml and 0.22 ± 0.13 mg/ml, respectively (Fig. 3).

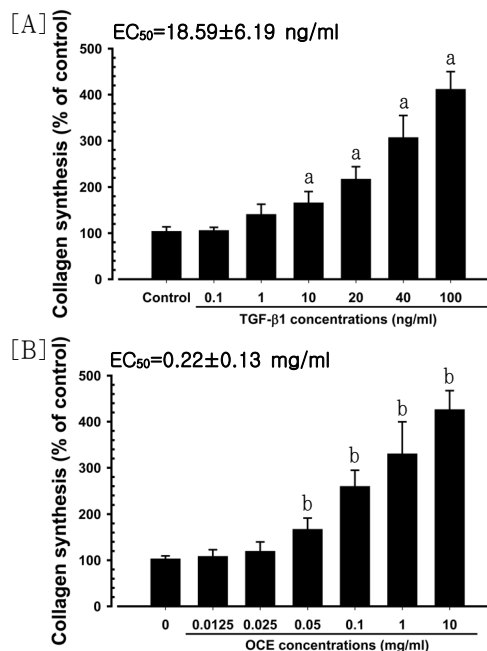


Fig. 3. Effects of TGF- β 1[A] and OCE[B] on the fibroblast collagen type I synthesis.

a : $p < 0.01$ as compared with control by LSD test
 b : $p < 0.01$ as compared with control by MW test

2) Hyaluronidase inhibitory activity

Significant ($p < 0.01$) increases of the inhibitory activities against hyaluronidase were detected from 0.025 mg/ml concentration of OA and OCE treated samples, and accordingly, IC_{50} of OA and OCE on the hyaluronidase activity were detected as 0.10 ± 0.07 and 0.44 ± 0.44 mg/ml, respectively (Fig. 4).

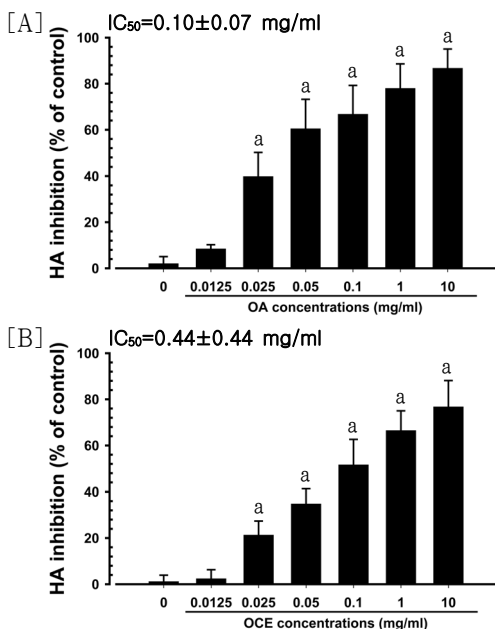


Fig. 4. Hyaluronidase inhibitory activity of OA[A] and OCE[B].
a : p<0.01 as compared with control by LSD test

3) Elastase inhibitory activity

Elastase activities were significantly (p<0.01) inhibited by treatment of PP and OCE from 1.25 ng/ml and 0.025 mg/ml concentrations, and IC₅₀ of PP and OCE on the elastase activity were detected as 10.46±4.90 ng/ml and 0.31±0.30 mg/ml, respectively (Fig. 5).

4) Collagenase inhibitory activity

Significant (p<0.01) increases of the inhibitory activities against collagenase were detected from 0.025 mg/ml of OA and 0.05 mg/ml concentration of OCE treated samples, and IC₅₀ of OA and OCE on the collagenase activity were detected as 0.09±0.08 and 1.02±0.52 mg/ml, respectively (Fig. 6).

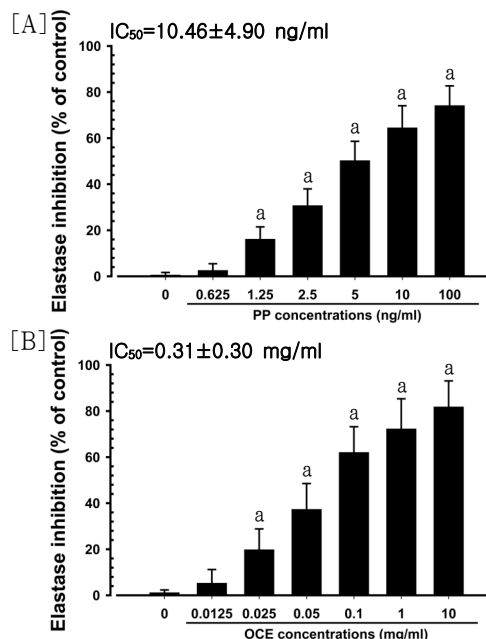


Fig. 5. Elastase inhibitory activity of PP[A] and OCE[B].
a : p<0.01 as compared with control by MW test

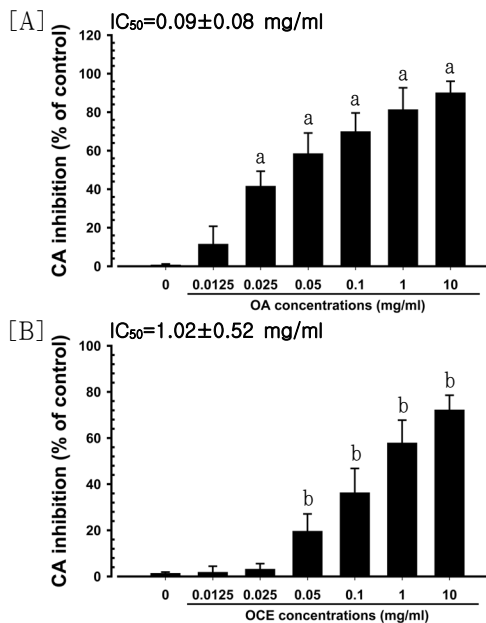


Fig. 6. Collagenase inhibitory activity of OA[A] and OCE[B].
a : p<0.01 as compared with control by LSD test
b : p<0.01 as compared with control by MW test

5) MMP-1 inhibitory activity

Significant ($p < 0.01$ or $p < 0.05$) increases of the inhibitory activities against MMP-1 were detected from 0.025 mg/ml of OA and 0.05 mg/ml concentration of OCE treated samples, and IC_{50} of OA and OCE on the MMP-1 activity were detected as 0.55 ± 0.41 and 2.51 ± 2.70 mg/ml, respectively (Fig. 7).

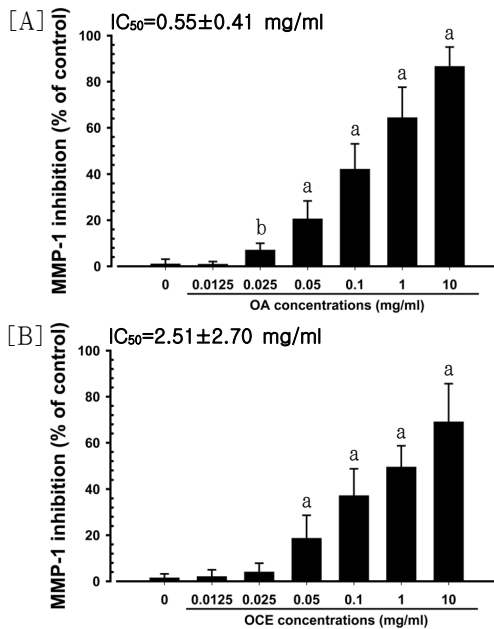


Fig. 7. MMP-1 inhibitory activity of OA[A] and OCE[B].

a : $p < 0.01$ and b : $p < 0.05$ as compared with control by MW test

3. Whitening effects

1) Tyrosinase inhibitory activity

Significant ($p < 0.01$) increases of the inhibitory activities against mushroom tyrosinase (MT) were detected from 20 μ g/ml of arbutin and 25 μ g/ml concentration of OCE treated samples, and IC_{50} of arbutin and OCE on the tyrosinase activity

were detected as 73.37 ± 22.54 and 96.13 ± 32.55 μ g/ml, respectively (Fig. 8).

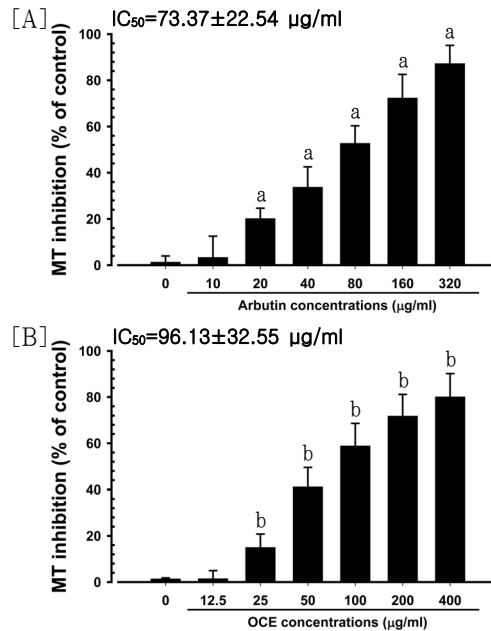


Fig. 8. MT inhibitory activity of arbutin[A] and OCE[B].

a : $p < 0.01$ as compared with control by LSD test

b : $p < 0.01$ as compared with control by MW test

2) Melanin formation (MF) of B16/F10 murine melanoma cell

Arbutin and OCE also significantly ($p < 0.01$) inhibited melanin product of the B16/F10 murine melanoma cell from 80 and 200 μ g/ml concentrations, and IC_{50} of arbutin and OCE on the melanin production were detected as 180.23 ± 75.75 and 579.05 ± 162.42 μ g/ml, respectively (Fig. 9).

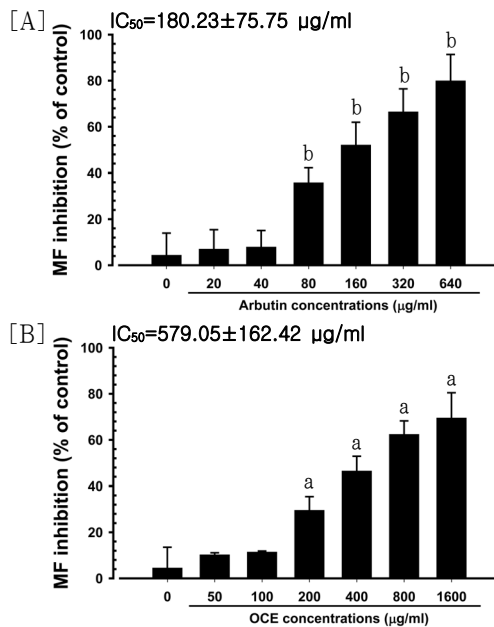


Fig. 9. MF inhibitory activity of arbutin [A] and OCE [B].
a : p<0.01 as compared with control by

LSD test
b : p<0.01 as compared with control by MW test

4. *In vivo* skin moisturizing effects

1) Changes on the body weights

There are no significant or meaningful changes on the body weight and gains during 7 days of experimental periods in all OCE treated mice as compared with vehicle control mice, respectively (Table 2, Fig. 10).

The body weight gains during 7 days of experimental periods in OCE 500, 250 and 125 mg/kg administered mice showed -3.03, -1.01 and 5.05% point changes as compared with vehicle control mice, respectively.

Table 2. Changes on the Body Weight Gains on the *In Vivo* Mouse Skin Moisturizing Assay

Groups		Body weights		Weight gains [B-A]
		First administration* [A]	Sacrifice* [B]	
Control	Intact vehicle	29.35±2.95	30.59±2.51	1.24±0.85
	500 mg/kg	29.63±1.82	30.83±2.02	1.20±0.56
OCE treated	250 mg/kg	29.80±2.35	31.03±2.19	1.23±0.49
	125 mg/kg	29.10±1.67	31.21±1.68	1.30±0.85

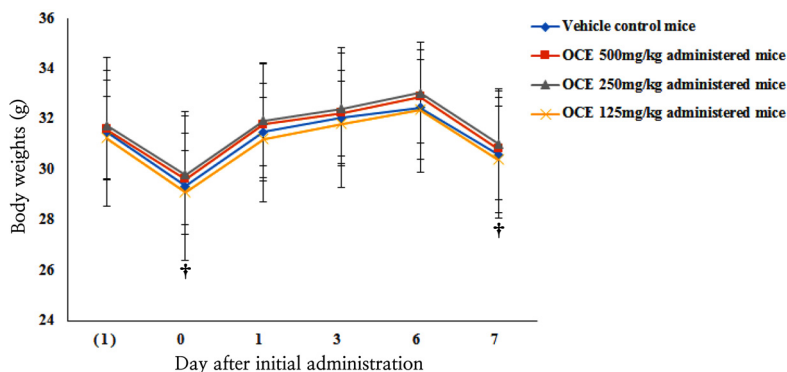


Fig. 10. Body weight changes on the *in vivo* mouse skin moisturizing assay. The day (1) means 1 day before initial administration of test materials. The day 0 means at first test material administration. All animals were overnight fasted before first test material administration and sacrifice, the day 7(†).

2) Effects on skin water contents

Significant ($p < 0.01$ or $p < 0.05$) increases of skin water contents were detected by oral treatment of all three different dosages of OCE, 500, 250 and 125 mg/kg, dose-dependently as compared with vehicle control mice at 24 hrs after last 7th administration, respectively (Fig. 11).

The skin water contents in OCE 500, 250 and 125 mg/kg administered mice showed 46.03, 29.31 and 17.49% point changes as compared with vehicle control mice, respectively.

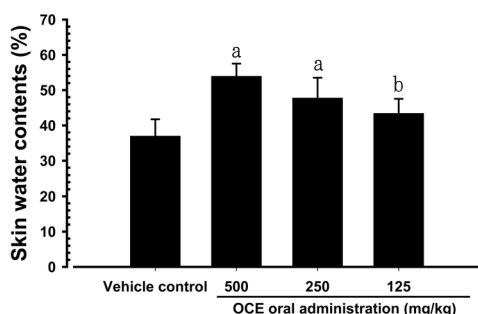


Fig. 11. Changes on the skin water contents on the *in vivo* mouse skin moisturizing assay.

a : $p < 0.01$ and b : $p < 0.05$ as compared with intact vehicle control by LSD test

IV. Discussion

Aging is a successive and natural phenomenon according to the passage of time. Skin aging is divided into intrinsic aging generated according to flow and photoaging due to ultraviolet rays, and the result of menopause or many outside stimuli working compositively^{23,24)}.

The skin is the biggest and most outward organization revealed the human body²³⁾. A remarkable difference should appear according to the external magnetic pole or management between the same ages. So skin care is important in modern society given the emphasis on appearance as individual competitiveness and a method to maintain self-satisfaction and friendly relationships²⁵⁾.

Especially, a woman's skin condition undergoes rapid change according to the stage of life such as adolescence, childbirth and menopause, and stress due to imbalance between social activities and housework²⁶⁾. In «Somun · Sanggocheonjinnon», the aging of women was recorded every seven years. And Kim et al studied women's skin aging in traditional Korean medicine from the point of view of color and gloss. So the book confirmed a decrease of in skin brightness from ages 28 ~ 35 and a prominent increase of red flags from ages 42 ~ 49^{3,4)}. These aesthetic skin changes can increase physical or psychological disorders and reduces quality of life for the aging woman, as well as cause stress and a vicious circle reducing skin elasticity and pigment variation^{23,24)}.

Under this social atmosphere, several studies on making professional products that not only protect skin but also perform functions like skin whitening, wrinkle protection and atopy were conducted by combining dermatology through the special dosage form method of construction²⁷⁾. Available ingredients

for functional foods or cosmetics for skin aging are too expensive, however, and exact or detailed pharmacological and side effects are not fully understood¹³⁾. Due to these factors, the search continues for affordable and effective ingredients with fewer side effects, especially natural antioxidants^{12,18)}.

OCE has the function of warming and supplying blood with a prescription combining *Samul-tang* and *Hwangryunhaedok-tang* while reducing fever. And OCE was recorded in *«Manbyunghoichun»* for the first time and this record was seen in *«Bugwaokcheock»*⁵⁾.

This prescription was used mainly in gynecology to stop bleeding caused by menorrhagia before the Qing Dynasty, but also extensively applied to skin disease, recurrent gutter wounds, Behcet's disease and diabetes. The study of skin diseases was the most common among studies on OCEs in Korea Japan and China. The following study was on gynecological disease⁵⁾.

OCE has the effect of removing heat and helping blood circulation; its effectiveness in treating gynecological and skin disease had been previously confirmed⁵⁾. So the study of this prescription was considered helpful to improve the skin of a menopause patient suffering from improper vasomotoricity symptoms, especially in the gynecological zone of the future. So we evaluated OCE's effects on skin regeneration, anti-wrinkle, and whitening and moisturizing.

The cytotoxicity of OCE was observed against human normal fibroblast cells and B16/F10 murine melanoma cells by MTT assay, and skin regeneration and anti-wrinkle effects were also observed through the assay of collagen type I synthesis by an EIA kit compared to TGF- β 1, hyaluronidase, collagenase and MMP-1 inhibitory assays compared to OA, and elastase inhibitory assay compared to PP. In addition, OCE's whitening effects were additionally measured by tyrosinase inhibitory assay and a melanin formation test in B16/F10 murine melanoma cells as compared to arbutin, and skin moisturizing effects were observed through a mouse skin water content test, respectively. In the effects of *in vivo* skin moisturizing, eight mice were prepared in each group, and the water content (%) in 2×3 cm of dorsal hair clipped back skin were measured by automated moisture analyzers balance 24 hrs after the seventh and final oral administration of vehicle, OCE 500, 250 and 125 mg/kg, in this study.

Cell growth inhibition assay using MTT is generally used in *in vitro* assay to detect cytotoxic activities in test materials, and widely used for basic safety screening of candidates²⁸⁾. In this assay, a favorable agent for skin protection should show lower cytotoxic effects on normal fibroblast and melanin cell lines²⁹⁾. In this study, OCE treatment showed no cytotoxic effects against human normal fibroblast and B16/F10 murine melanoma cells, from 1.25 to 500 mg/ml

concentrations, the lowest to highest concentrations tested in this study. This is considered direct evidence that OCE showed no cytotoxic effects on normal skin cells.

Type I collagen is the major structural protein in skin. The destruction of collagen is thought to underlie the appearance of aged skin and changes caused by chronic sun exposure^{24,30}. The formation of skin wrinkles is an inevitable process of human life involving the age-dependent decline of skin cell function²³. Several pieces of scientific evidence on skin wrinkling highlight the degradation of ECM, which is significantly associated with higher activity of dermal enzymes like hyaluronidase, collagenase, elastase and MMP-1 and formation of wrinkles^{15,31}. In this study, TGF- β 1 used as standard collagen synthesis reference showed potent growth of Type I pro-collagen synthesis ($EC_{50} = 18.59 \pm 6.19$ ng/ml), PP used as elastase inhibitory standard reference material showed potent inhibitory effects to elastase ($IC_{50} = 10.46 \pm 4.90$ ng/ml), and OA used as hyaluronidase, collagenase and MMP-1 inhibitory standard reference material showed potent inhibitory effects to hyaluronidase ($IC_{50} = 0.10 \pm 0.07$ mg/ml), collagenase ($IC_{50} = 0.09 \pm 0.08$ mg/ml) and MMP-1 ($IC_{50} = 0.55 \pm 0.41$ mg/ml), respectively. In addition, OCE also showed concentration-dependent increases of Type I collagen synthesis on fibroblasts ($EC_{50} = 0.22 \pm 0.13$ mg/ml) up to 500 mg/ml concentrations and favorable inhibitions to hyaluronidase

($IC_{50} = 0.44 \pm 0.44$ mg/ml), elastase ($IC_{50} = 0.31 \pm 0.30$ mg/ml), collagenase ($IC_{50} = 1.02 \pm 0.52$ mg/ml) and MMP-1 ($IC_{50} = 2.51 \pm 2.70$ mg/ml). These results form direct evidence that OCE has adequate anti-wrinkle effects and can be an ingredient in anti-skin aging food or cosmetics.

Melanin is the main component determining skin color and produces melanin pigments up to 10 percent of cells in the epidermis' innermost layer³². The major role of melanin is to protect the skin from the damaging effects of ultraviolet radiation³². Melanin biosynthesis, or melanogenesis, is a well-known physiological response of human skin upon exposure to ultraviolet light and other stimuli. Melanogenesis is regulated by enzymes such as tyrosinase and tyrosinase-related protein (TRP)-1 and TRP-2²⁴. The inhibition of tyrosinase activity is the most common approach to achieve whiter skin as it is the key enzyme that catalyzes the rate-limiting step of melanin biosynthesis²⁴. In addition, the B16/F10 murine melanoma cell line was used because it produces melanin, is known to contain tyrosinase, which is associated with melanogenesis, responds to α -MSH activation and is easy to culture *in vitro*³³. The results of our study showed that arbutin used as a standard whitening reference showed potent inhibitory effects to mushroom tyrosinase ($IC_{50} = 73.37 \pm 22.54$ μ g/ml), and melanin produces B16/F10 murine melanoma cells ($IC_{50} = 180.23 \pm 75.75$

µg/ml), respectively. In addition, OCE also showed concentration-dependent inhibitions to tyrosinase ($IC_{50} = 96.13 \pm 32.55$ µg/ml) and melanin produces B16/F10 murine melanoma cells ($IC_{50} = 579.05 \pm 162.42$ µg/ml), so these results are direct evidence that OCE has adequate whitening effects.

Normal layers of human keratin maintain skin moisture: they contain 10~20 percent water but a decrease in water content in keratin layers accelerate aging processes like wrinkle formation and itching¹¹⁾. Due to these facts, keratin layers must retain enough moisture to maintain healthy and elastic skin, even in dry conditions³⁴⁾. The effects of skin moisturizing are easily detectable by water content through animal skin or human pilot study^{18,19)}. Significant ($p < 0.01$ or $p < 0.05$) and dose-dependent increases in skin water content were detected in OCE administered mouse dorsal back skins compared to vehicle control mice at 24 hrs after the seventh and last treatment, suggesting OCE has potent skin moisturizing effects and can be a potent ingredient for cosmetics in this study. No significant or meaningful changes to body weight were detected in all three doses of OCE administered mice, 500, 250 and 125 mg/kg compared to vehicle control mice; consequently, administration of OCE did not influence body weight gains over seven days of continuous oral administration in the current results. All mice used in this experiment showed normal body weight and gain throughout

the seven-day experimental period, resulting in age-matched normal reference ICR mice³⁵⁾.

The OCE used consisted of eight herbs - *Angelicae Gigantis Radix*, *Rehmanniae Radix*, *Paeoniae Radix*, *Cnidii Rhizoma*, *Scutellariae Radix*, *Coptidis Rhizoma*, *Phellodendri Cortex* and *Gardeniae Fructus*⁵⁾ - and each had various active ingredients.

Angelicae Gigantis Radix contained Decursinol, 7-demethyl-suberone, Umbelliferone and other ingredients, but the principal pharmacological compound was known to be caused by Decursin. And Decursin appeared to have the effects of anti-wrinkle and whitening in the research of Yoo et al³⁶⁾. *Rehmanniae Radix* contained Catalpol, Verbascose and Mannitol, and Catalpol was reported as having an anti-oxidation effect³⁷⁾. *Paeoniae Radix* had paeoniflorin in which the anti-wrinkle effect was proven with an effective component³⁸⁾. *Cnidii Rhizoma* was found in many refined oil materials. And the anti-wrinkle effect of the hot water extract was proven³⁹⁾. Baicalein, one among various flavonoids of *Scutellariae Radix*, was known to dilute MMP-1 activity with anti-oxidant in keratinocyte activity⁴⁰⁾. Berberine, palmatine and others of *Coptidis Rhizoma* and *phellodendri cortex* were known to hinder melanogenesis and give off a whitening effect⁴¹⁾. Finally, *Gardeniae fructus* was also reported to have an anti-oxidation effect⁴²⁾. So screening of active biological compounds in individual herbs constituting OCE should be conducted

with more detail mechanisms and studies on the skin protective efficiency of *in vivo*.

Considering the results of this experiment, the suggestion is that OCE showed favorable and sufficient effects in skin regeneration, anti-wrinkle, whitening and moisturizing to serve as a predictable ingredient to fight skin aging. In addition, standard reference materials used in this experiment - Arbutin, TGF- β 1 OA, or PP - also showed favorable effects in skin regeneration, anti-wrinkle or whitening and had a range of reference values, suggesting the experiment protocol and results of the study were acceptable. Yet more detail mechanisms and studies on the skin protective efficiency of *in vivo* should be conducted with the screening of active biological compounds in individual OCE herbs.

V. Conclusion

In this study, we reached the following results:

1. OCE showed no cytotoxic effects on human normal fibroblast and B16/F10 murine melanoma cells.
2. The Type I collagen synthesis significantly

increased on TGF- β 1 and OCE-treated fibroblasts.

3. Hyaluronidase activities were significantly inhibited on OA and OCE-treated samples that were concentration dependent.
4. Elastase activities were significantly inhibited by the treatment of PP and OCE that was concentration dependent.
5. Higher inhibitory activity against collagenase was significantly detected in OA and OCE-treated samples that were concentration dependent.
6. MMP-1 activities were significantly inhibited by treatment of OA and OCE that was concentration dependent.
7. Arbutin and OCE significantly inhibited mushroom tyrosinase activities that were concentration dependent.
8. Significant increases in skin water content were detected by oral treatment of OCE dependent on dose compared to vehicle control mice.

According to these results, OCE is expected to serve as a functional ingredient for fighting and slowing skin aging.

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국문초록

목적: 본 연구에서는 한의학에서 다양한 피부질환과 대사질환에 빈번히 사용되고 있는 온청음 물 추출 동결건조물(수율=13.82%)의 피부 노화 개선 효과 평가의 일환으로 세포독성, 피부재생, 주름개선, 미백 및 보습 효과를 각각 평가하였다.

방법: 본 연구에서는 human normal fibroblast(CRL-2076) 및 B16/F10 murine melanoma(CRL-6475) 세포에 대한 온청음의 세포독성을 MTT(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium Bromide) 방법으로 평가하였으며, 피부 재생 및 주름 개선 효과를 transforming growth factor(TGF)- β 1와 비교한 fibroblast의 collagen type I 합성능, phosphoramidon disodium salt(PP)와 비교한 elastase 활성 억제, oleanolic acid(OA)와 비교한 hyaluronidase, collagenase 및 matrix metalloproteinase(MMP)-1 활성 억제를 통해 각각 평가하였고, 미백효과를 B16/F10 murine melanoma cells의 melanin 생성 억제 정도 및 tyrosinase 활성 억제를 통해 arbutin과 비교 평가하였으며, 모든 실험은 OCE의 농도별로 군을 나누어 농도에 따른 효과의 변화를 함께 분석하였다. 보습효과는 흰쥐의 피부 수분함량 변화를 통해 평가하였다.

결과: 본 실험의 결과, 온청음은 human normal fibroblast 및 B16/F10 murine melanoma 세포에 대해 의미 있는 세포독성을 나타내지 않았으며, fibroblast의 collagen type I 합성을 증가시켰고, 세포외 기질의 파괴에 관여한다고 알려진 hyaluronidase, elastase, collagenase 및 MMP-1 활성을 억제하였으며, 피부의 색을 결정하는 melanin의 생성에 관여하는 tyrosinase의 활성 및 B16/F10 murine melanoma cells의 melanin 생성을 억제하는 것으로 관찰되었다. 이 반응의 효과들은 모두 농도에 비례하여 증가하였고, 이와 함께 정상 매체 대조군에 비해 흰쥐의 피부 수분 함량이 세 용량의 온청음 경구 투여군 모두에서 투여용량 의존적으로 의미 있는 증가를 보였다.

결론: 이상의 결과에서, 온청음은 세포 독성 없이 비교적 우수한 피부 재생, 주름개선, 미백 및 보습 효과를 나타내는 것으로 관찰되어, 차후 피부 노화 억제 개선제 또는 기능성 화장품의 주요 소재로서 그 가치가 매우 높을 것으로 판단되나, 금후 개별 구성 약재 각각에 대한 효능 및 생리활성을 나타내는 화학성분의 검색과 더불어 다양한 방면으로 기전적인 연구와 피부 보호 효과에 대한 *in vivo* 평가를 체계적으로 수행해야 할 것으로 판단된다.

중심단어: 온청음, 피부재생, 항노화, 미백, 보습

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