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청상방풍탕 열수 추출물의 피부재생, 주름개선, 미백, 보습 효과 및 세포독성 평가

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ABSTRACT

Skin Regeneration, Anti-wrinkle, Whitening and Moisturizing Effects of *Cheongsangbangpung-tang* Aqueous Extracts with Cytotoxicity

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Objectives: The present study is to observe the skin-regeneration, anti-wrinkle, whitening and skin moisturizing effects of *Cheongsangbangpung-tang* (CSBPT) with cytotoxicity.

Methods: In the present study, cytotoxicity of CSBPT lyophilized aqueous extracts (yield=18.71%) was experimented against human normal fibroblast cells and B16F10 murine melanoma cells by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT) assay, and skin regeneration and anti-wrinkle effects were also showed through the assay of collagen type I synthesis by an enzyme immunoassay (EIA) kit as comparing with transforming growth factor (TGF)-β1, hyaluronidase, collagenase and matrix metalloproteinase (MMP)-1 inhibitory assays as comparing with oleanolic acid (OA), and elastase inhibitory effects as comparing with phosphoramidon disodium salt (PP). In addition, whitening effects of CSBPT were observed by tyrosinase inhibitory assay and melanin formation test in B16/F10 melanoma cells as comparing with arbutin, and skin moisturizing effects were measured through mouse skin water contents test, respectively.

Results: No CSBPT treatment related cytotoxic effects were demonstrated against human normal fibroblast cells and B16/F10 murine melanoma cells. CSBPT concentration-dependent increased collagen type I synthesis at human normal fibroblast cells. It also effectively suspreessed hyaluronidase, collagenase, elastase and MMP-1 activities, which were enzymes that related to declining of ECM and formation of wrinkle. CSBPT supressed B16/F10 melanoma cells's melanin productions with tyrosinase activity, which was an enzyme connected with melanin formation, and dose-dependent and significant increases of skin water contents were detected in CSBPT treated mouse skin as compared with vehicle control skins.

Conclusions: CSBPT showed favorable and enough skin regeneration, anti-wrinkle, whitening and skin moisturizing effects at least in a condition of this experiment. However, more detail mechanism and in vivo skin protective efficacy studies should be conducted in future with the screening of the biological active compounds in individual herbs of *Cheongsangbangpung-tang*.

Key Words: *Cheongsangbangpung-tang*, Skin Regeneration, Anti-wrinkle, Whitening, Moisturizing

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Ⅰ. Introduction

Throughout entering the age and type of changes in intangible that appear on the skin, called skin aging. But although completely irreversible, because it is not a biological law that is transferred to the will of mankind, slowing the modern scientific and technological progress of aging and applications, and therefore the quality of life also improved by more youthful that beauty, as well as to extend the human lifespan it may be effective[1].

Improvement of facial skin aging is a major concern for aging in middle-aged women overcome anxiety. It has a positive effect on overcoming their negative self-esteem, such as degradation, unworthiness, lack of confidence. In addition, focusing on youth and their psychological satisfaction has been recognized as an important means of restoring confidence in the social pressures on the values physical beauty of women[2].

Cheongsangbangpung-tang is the first time prescriptions contained in ≪gogeumuigam≫ in the Ming dynasty of China, and was mainly used for treating facial comedones. As this fact, possible favorable improvement effects of CSBPT on acne have been reported by other researchers[3,4]. And antioxidant activity was also demonstrated[5].

In the present study, cytotoxicity of CSBPT was experimented with human normal fibroblast cells, B16F10 murine melanoma cells by MTT (3-(4,5-dimethylthiazol -2-yl)-2,5-diphenyltetrazolium Bromide) assay, skin regeneration and anti-wrinkle effects were also experimented through the collagen type I synthesis assay by an EIA kit as comparing with TGF-β1, hyaluronidase, MMP-1 inhibitory assays and collagenase as comparing with OA, and elastase inhibitory effects as comparing with PP. In addition, whitening effects of CSBPT were additionally observed by tyrosinase inhibitory assay, test of melanin formation in B16/F10 melanoma cells as comparing with arbutin, and skin moisturizing effects were measured through mouse skin water contents test.

As a result of the experiment, it is thought that CSBPT showed favorable skin-regeneration, anti-wrinkle, whitening and skin moisturizing effects. So we report the experiment through this paper.

Ⅱ. Materials and Methods

1. Preparations and administration of test materials

Individual 12 types of herbs consisted of *Cheongsangbangpung-tang* were purchased from local herb shop (Jecheonhanbangyakcho, Jecheon, Korea) under inspection of herbologist (Dr. Park KY), and the compositions of *Cheongsangbangpung-tang* and product regions were writed in Table 1. Total 30.4 g of appropriate proportions of individual herbs consisted of *Cheongsangbangpung-tang* were boiled in 1,000 ml of distilled water for 4 hrs and 3 times at 60℃, and

vaporized making use of automated round flasked evaporator (Eyela N-1110, Tokyo, Japan), next completely lyophilized making use of a programmable freeze dryer (FDB-5503, Operon, Kimpo, Korea). Total 5.69 g (yield=18.71%) of lyophilized *Cheongsangbangpung-tang* aqueous extracts were acquired, and were laid in a refrigerator at -20℃ to preserve from light and humidity before used, and some samples of CSBPT (CSBPT2013KDC) were placed in the hortus siccus of the Medical Research Center for Globalization of Herbal Formulation, Daegu Haany University (Gyeongsan, Korea). Colorless to white powder of arbutin (Sigma-Aldrich, St. Louise, MO, USA), white powder of TGF-β1 (R&D Systems, Minneapolis, MN, USA), white to slightly yellow solid of PP (Sigma, St. Louise, MO, USA), white solid of OA (Sigma, St. Louise, MO, USA) were used standard references for skin-regeneration, anti-wrinkle or whitening effects, respectively. They were also stored at -20℃ in a refrigerator for protecting from light and humidity in this experiment.

Table 1. Composition of CSBPT Used in This Study

Herbs	Scientific name	Korean name	Produce region	Amounts (g)
Saposhnikoviae Radix	Saposhnikovia divaricata Schischkin	防 風	Hwasoon, Chunnam	4.00
Forsythiae Fructus	Forsythia viridissima Lindley	連 翹	Youngju, Gyeongbuk	3.20
Atractylodis Rhizoma Alba	Atractylodes japonica Koidzumi	白 芷	Hwasoon, Chunnam	3.20
Platycodi Radix	Platycodon grandiflorum A. De Candolle	桔 梗	Jecheon, Chungbuk	3.20
Scutellariae Radix	Scutellaria baicalensis Georgi	黃 芩	Jeongseon, Gangwon	2.80
Cnidii Rhizoma	Cnidium officinale Makino	川 芎	Youngyang, Gyeongbuk	2.80
Schizonepetae Spica	Schizonepeta tenuifolia Briquet	荊 芥	Andong, Gyeongbuk	2.00
Gardeniae Fructus	Gardenia jasminoides Ellis	梔 子	Jindo, Chunnam	2.00
Coptidis Rhizoma	Coptis japonica Makino	黃 蓮	China	2.00
Aurantii Fructus Immaturus	Citrus aurantium Linne	只 殼	China	2.00
Menthae Herba	Mentha arvensis Linne var. piperascens Malinvaud ex Holmes	薄 荷	Andong, Gyeongbuk	2.00
Glycyrrhizae Radix	Glycyrrhiza uralensis Fischer	甘 草	Jecheon, Chungbuk	1.20
Total	12 types			30.40

2. Cytotoxicity assay in a monolayer culture

Human normal fibroblast cells (CRL-2076; ATCC, Manassas, VA, USA), B16/F10 murine melanoma cells (CRL-6475; ATCC, Manassas, VA, USA) were incubated in Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich, St. Louise, MO, USA) retaining 10% bovine fetal 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℃ in humidity of 5% CO2. Next the cells were incubated with 0.05% trypsin-0.53mM EDTA (Sigma-Aldrich, St. Louise, MO, USA) after changing with a fresh medium every 2 or 3 days. In B16/F10 murine melanoma cells, 2 mM L-glutamine (Sigma-Aldrich, St. Louise, MO, USA) were additionally provided in culture medium. Human fibroblast cells, B16/F10 murine melanoma cells were implanted in 24-well plates at a density of 1×105 cells per well and incubated at 37℃ in 5% CO2. After one day, a fresh medium containing 2% serum was added and the cells were in a CO2 incubator at 37℃ in the presence of samples (1.25, 2.5, 5, 10, 100 and 500 mg/ml of CSBPT) for 24 hrs, before being dealt with 100 μl of 2.5 mg/ml of MTT (Sigma-Aldrich, St. Louise, MO, USA). After then The cells incubated at 37℃ for an additional 4 hrs. The medium containing MTT was abandoned, and MTT formazan which had been produced before was extracted with 1 ml of DMSO. The absorbance was read at 570 nm with a reference wavelength of 650 nm with a micro plate reader (Tecan, Männedorf, Switzerland). The cell viability is calculated according to the following equation [1].

Equation [1]. Cell viability (%) = OD570(sample)/OD570(control)×100

OD570 (sample) is the absorbing power of the treated cells at 570 nm.

OD570 (control) is the absorbing power of the negative control at 570 nm.

3. Anti-wrinkle effects

Anti-wrinkle effects of test samples were also experimented through the collagen type I synthesis assay[6], the inhibition of hyaluronidase[7], elastase[8], collagenase[9] and MMP-1[10] in this study.

1) Collagen type I synthesis assay by an EIA kit

Anti-wrinkle effects of test samples were experimented through the collagen type I synthesis assay[6] in this study. Simply, fibroblast cells were vaccinated into 24-well plates (5×105 cells/well) and cultured for 24 hrs. After then the culture medium was altered to non serum IMDM (Iscove's modified Dulbecco's medium; Sigma-Aldrich, St. Louise, MO, USA) and cultured for 24 hrs inclusion 0.0125, 0.025, 0.05, 0.1, 1 and 10 mg/ml of test samples (CSBPT) or 0.1, 1, 10, 20, 40 and 100 ng/ml of TGF-β1. The control group was cultured without a compound. After cultivating, the supernatant fluids was collected from each well, and

the quantity of pro-collagen type I was observed with a pro-collagen type I C peptide assay kit (Takara Bio, Tokyo, Japan), read at 450 nm with a micro plate reader. The pro-collagen synthesis was calculated on following equation [2] and the results were notified in terms of EC50 (the concentration which the percentage increases of collagen type I synthesis was 200%).

Equation [2]. Pro-collagen synthesis (%) = OD450(sample)/OD450(control)×100

OD450 (sample) is the absorbing power of the treated cells at 450 nm.

OD450 (control) is the absorbing power of the negative control at 450 nm.

2) Hyaluronidase inhibitory assay

Hyaluronidase inhibitory assay was performed according to previously reported methods[7]. Hyaluronidase respond with the substrate hyaluronic acid to discharged N-acetyl glucosamine. With all the inhibitors, the release of N-acetyl glucosamine is decreased and it is watched by measuring the absorbance at 600 nm. The inhibitory activity of the CSBPT (0.0125, 0.025, 0.05, 0.1, 1 and 10 mg/ml) was compared to OA as a standard under completely identical experimental conditions. A 600 nm value of whole undigested hyaluronic acid was set on 100%. The Optical density at 600 nm was observed with a 96 well microplate reader after 15 mins (Tecan, Männedorf, Switzerland) and each sample's hyaluronidase inhibitory activity was calculated as follow equation [3].

Equation [3]. Hyaluronidase inhibitory activity (%) = 100-{[(ODs+ODc)/ODc]×100}

ODs is the absorbing power of the sample.

ODc is the absorbing power of the vehicle treated control at 600 nm.

The results are stated in terms of IC50 (the concentration which the percentage inhibition of hyaluronidase activity was 50%).

3) Elastase inhibitory assay

Elastase inhibitory assay was discharged by observing the release of p-nitro aniline come from proteolysis of N-succinyl-(Ala)3-p-nitroanilide by human leucocyte elastase (Sigma-Aldrich, St. Louise, MO, USA)[8] in the presence or absence of the CSBPT (12.5, 25, 50, 100, 200 and 400 μg/ml) or PP (0.625, 1.25, 2.5, 5, 10 and 100 ng/ml) as a norm under correctly the equal experimental conditions. The absorbance was measured at 410 nm with a 96 well micro plate reader and each sample's elastase inhibitory activity was calculated as follow equation [4]. The results are stated in terms of IC50 (the concentration which elastase inhibitory activity's percentage was 50%).

Equation [4]. Elastase inhibitory activity (%) = 100-[(ODs/ODc)×100]

ODs is the absorbing power of the experimental sample.

ODc is the absorbing power of the vehicle treated control at 410 nm.

4) Collagenase inhibitory assay

Collagenase inhibitory assay was discharged according to previously reported methods[9]. 0.15 ml of collagenase (1 mg/ml; Sigma-Aldrich, St. Louise, MO, USA) was

attached to the mixed solutions made up 2 mM 4-phenylazobenzyloxycarbonyl-pro-leu-gly-pro-d-ar (Sigma-Aldrich, St. Louise, MO, USA) 0.25 ml and 0.1 ml of CSBPT (0.0125, 0.025, 0.05, 0.1, 1 and 10 mg/ml) in 0.1 M Tris-HCl buffer (pH 7.5) and responded for 20 mins at 37℃. After this, the responds were halted by superunducing 6% citric acid (Daejung, Seoul, Korea) 0.5 ml. The absorbance was measured at 320 nm with a UV/Vis spectrophotometer after adding ethyl acetate (Sigma-Aldrich, St. Louise, MO, USA) 1.5 ml and each sample's collagenase inhibitory activity was calculated as following equation [5].

Equation [5]. Collagenase inhibitory activity (%) = 100-[(ODs/ODc)×100]

ODs is the absorbing power of the experimental sample.

ODc is the absorbing power of the vehicle treated control at 320 nm.

The results are stated in terms of IC50 (the concentration which collagenase inhibitory activity's percentage was 50%). OA (0.0125, 0.025, 0.05, 0.1, 1 and 10 mg/ml) was used as a norm under correctly the equal experimental conditions.

5) MMP-1 inhibitory assay

MMP-1 inhibitory assay was experimented over fluorescence microplate as described previously[10] with slight modification. Simply, 20 μl of type-I collagen (substrate; Sigma-Aldrich, St. Louise, MO, USA) was blended with 80 μl of each of diluted CSBPT or OA (0.0125, 0.025, 0.05, 0.1, 1 and 10 mg/ml) as a norm under correctly the equal experimental conditions. Then 100 μl of diluted (0.2 U/ml) MMP-1 (Sigma-Aldrich, St. Louise, MO, USA) was added to each well and the plate was cultured at room temperature for 1 hr to 2 hrs and protected from light. Fluorescence was measured at excitation maxima at 495 nm and emission maxima at 515 nm. All dilutions were made up with a 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 group used in this experiment was a buffer with the substrate and the inhibitors without each sample's MMP-1. MMP-1 inhibitory activity was calculated as following equation [6].

Equation [6]. MMP-1 inhibitory activity (%) = 100-[(ODs/ODc)×100]

ODs is the absorbing power of the experimental sample.

ODc is the absorbing power of the vehicle treated control at 515 nm.

The results are stated in terms of IC50 (the concentration which MMP-1 inhibitory activity's percentage was 50%).

4. Whitening effects

The whitening effects of CSBPT were measured by tyrosinase inhibitory assay[11] and melanin formation test in B16/F10 melanoma cells[12] in the present study.

1) Tyrosinase inhibitory assay

Tyrosinase inhibitory assay was experimented as described previously[11]. Simply, aliquots (0.05 ml) of CSBPT (12.5, 25, 50, 100, 200 and 400 μg/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 incubated at 25℃ for 10 mins. Then, 0.05 ml of a mushroom tyrosinase aqueous solution (333 U/ml; Sigma-Aldrich, St. Louise, MO, USA) was finally added to the mixture. This solution was immediately observed for the dopachrome formation by admeasuring the linear increase in OD at 475 nm with a UV/V is spectrophotometer and each sample's tyrosianse inhibitory activity was calculated as the following equation [7]. The results are stated in terms of IC50 (the concentration which tyrosinase inhibitory activity's percentage was 50%). Arbutin (10, 20. 40, 80, 160 and 320 μg/ml) was used as a norm under correctly the equal experimental conditions.

Equation [7]. Tyrosinase inhibitory activity (%) = 100-[(ODs/ODc)×100]

ODs is the absorbing power of the experimental sample.

ODc is the absorbing power of the vehicle treated control at 475 nm.

2) Melanin formation test in B16/F10 melanoma cells

The B16/F10 murine melanoma cells were reaped by trypsinization when they were about 70% confluent, calculated with a hemocytometer and seeded in the wells of the cell culture plates at an appropriate numbers for next experiments. Melanin content was observed as described previously[12] with slight changing. The B16F10 melanoma cells were planted with 2×105 cells/well in 3 ml of medium in 6-well culture plates and cultured overnight for allow cells to attach. The cells were exposed to various concentrations of CSBPT (50, 100, 200, 400, 800 and 1,600 μg/ml) for 72 hrs with or without 100 nm alpha-melanocyte stimulating hormone (α-MSH, Sigma-Aldrich, St. Louise, MO, USA). After the treatment, the cells were cleaned with PBS and dissolved with 800 μl of 1 N NaOH (Merck, Darmstadt, Germany) retaining 10% DMSO (Sigma-Aldrich, St. Louise, MO, USA) for 1 hr at 80℃. The absorbance at 400 nm was experimented using a micro plate reader. Each samples's melanin produce inhibitory activity was calculated as the following equation [8]. The results are stated in terms of IC50 (the concentration which melanin production inhibitory's percentage was 50%). Arbutin (20, 40, 80, 160, 320 and 640 μg/ml) was used as a norm under correctly the equal experimental conditions.

Equation [8]. Melanin produce inhibitory activity (%) = 100-[(ODs/ODc)×100]

ODs is the absorbing power of the experimental sample.

ODc is the absorbing power of the α-MSH treated control at 400 nm.

5. in vivo skin moisturizing effects

Skin moisturizing effects of experimental materials were experimented by the variations of mouse skin water contents[13-5] in this study.

1) Animal and husbandry

41 male SPF/VAF Outbred CrljOri:CD1[ICR] mice (6-wks old upon receipt, OrientBio, Seungnam, Korea) were used in this

experiment after 7 days of acclimatization. Animals were assigned 4 to 5 per polycarbonate cage in a controlled room by temperature (20-25℃) and humidity (40-45%). Light : dark cycle was 12 hrs : 12 hrs. It is supplied free to access normal rodent pellet diet during acclimatization. After acclimatization, eight mice per group were chosen based on the body weights (Mean 31.34±2.21 g, ranged in 27.50-34.70 g), and divided into four groups - vehicle control, CSBPT 500, 250 and 125 mg/㎏ orally administered groups. All laboratory animals were cared following to the national regulations of the using and well-being of laboratory animals, and approved by the Institutional Animal Care and Use Committee in Daegu Haany University (Gyeongsan, Korea) before animal experiment [Approval No DHU2015-032 2015. 04. 13]. All animals were overnight fasted before initial administration and sacrifice for 18 hrs (water was not restricted) to reduce entitative differences from feeding in the current study.

2) Treatment

CSBPT were lyzed 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 gavages using a zonde added to 1 ml syringe. In vehicle control, only distilled water was injected orally instead of CSBPT solutions in this experiment.

3) Body weight measurement

Changing of body weight was observed at 1 day before initial test article management, the day of first test material management, 1, 3, 6 and 7 days after initial vehicle or CSBPT management using an automatic electronic balance (Precisa Instrument, Zuerich, Switzland). To reduce the entitative differences, the body weight increasing during 7 days of experimental periods were calculated according to the following equation [9]. In addition, compared to vehicle controls, percent-point changes were worked out to help understanding the effect of test materials according to the following equation [10] as described previously[16].

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

4) Skin water content measurement

24 hrs after last 7 th administration of vehicle or CSBPT solutions, 2×3 cm of hair clipped dorsal skin samples were collected, and skin water contents (%) were calculated by automated moisture analyzers balance (MB23, Ohaus, Pine Brook, NJ, USA) as described previously[13]. In addition, compared to vehicle controls, percent-point changes were worked out to help understanding of the effect of test materials according to the following equation [10] as described previously[16].

Equation [10]. Percent-point changes compared with vehicle control (%) = [((CSBPT administration groups data -vehicle treated control data)/vehicle treated control data)×100]

6. Statistical analyses

In vitro data were showed as mean± standard deviation of 5 independent experiments and skin moisture was calculated as mean±standard deviation of eight mice skins at each time. Several comparison tests were performed for different dose groups. Homogeneity of variance was examined using the Levene test[17]. If the Levene test had no significant deviations in variance homogeneity, the obtain data from one way ANOVA test and the least-significant differences multi-comparison (LSD) test were analyzed to determine which group comparison pair was significantly different. In case of important deviations from homogeneity of variance was observed at Levene test, the Kruskal-Wallis H test, non-parametric comparison test, was conducted. When significant differences were observed in the Kruskal-Wallis H test, a Mann-Whitney U (MW) test was performed to determine the specific pair of group comparisons[18]. Differences were considered significant at $P<0.05$. EC50 or IC50 values in each in vitro assay were measured by Probit methods and statistical analysis was performed using SPSS for Windows (Release 14.0K, SPSS Inc., Chicago, IL, USA)[13].

Ⅲ. Results

1. Cytotoxic effects

1) Against human normal fibroblast cells

No CSBPT treatment related cytotoxic effects, the changes in the human normal fibroblast cell survivals, were demonstrated from 1.25 to 500 mg/ml concentrations, from lowest to highest concentrations tested in this experiment (Fig. 1).

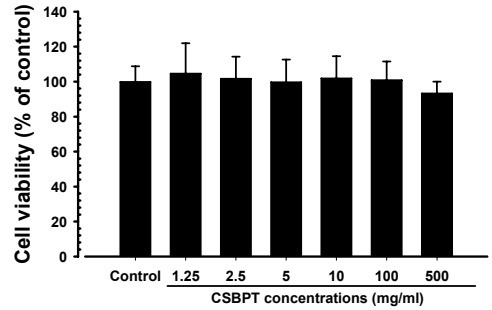

Fig. 1. Effects of CSBPT on the human normal fibroblast cell viabilities.

2) Against B16/F10 murine melanoma cells

Changes against the B16/F10 murine melanoma cell survivals were not showed in all six concentrations (from 1.25 to 500 mg/ml) of CSBPT treated cells as compared as non-treated vehicle control. This experiment suggested that there is no cytotoxicity of test samples on B16/F10 murine melanoma cells (Fig. 2).

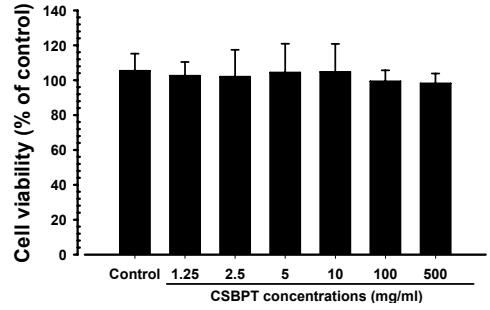

Fig. 2. Effects of CSBPT on the B16/F10 murine melanoma cell viabilities.

2. Skin regeneration and anti-wrinkle effects

1) Effect on the collagen type I synthesis

The collagen type I synthesis's attended ($p<0.01$ or $p<0.05$) increasing were detected from 1 ng/ml and 0.025 mg/ml concentration of TGF-β1 and CSBPT treated fibroblasts, and accordingly, EC50 of TGF-β1 and CSBPT on the type I collagen synthesis are detected as 18.93±8.72 ng/ml and 0.10±0.06 mg/ml (Fig. 3).

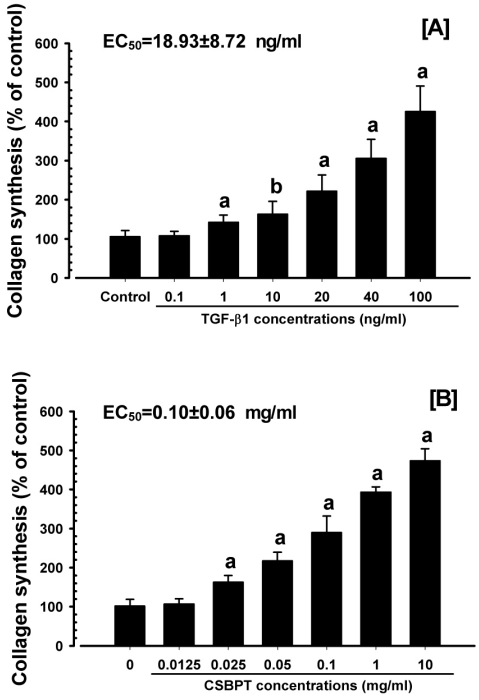

Fig. 3. Effects of TGF-β1 [A] and CSBPT [B] on the fibroblast collagen type I synthesis.
a : $p<0.01$ and b : $p<0.05$ compared with control by MW test

2) Hyaluronidase inhibitory activity

Hyaluronidase inhibitory activity's attended ($p<0.01$) increasing were detected from 0.025 mg/ml concentration of OA and CSBPT treated samples, and accordingly, IC50 of OA and CSBPT on the hyaluronidase activity are detected as 0.11±0.09 and 0.13±0.06 mg/ml (Fig. 4)

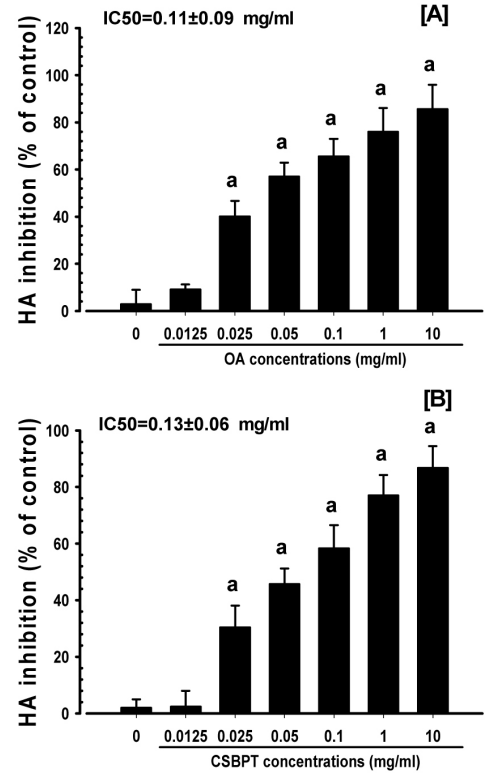

Fig. 4. Hyaluronidase inhibitory activity of OA [A] and CSBPT [B].
a : $p<0.01$ compared with control by LSD test

3) Elastase inhibitory activity

Elastase inhibitory activity was attended ($p<0.01$ or $p<0.05$) inhibited compared with treatment of PP and CSBPT from 1.25 ng/ml and 0.025 mg/ml concentrations, and accordingly, IC50 of PP and CSBPT on the elastase activity are detected as 11.76±7.92 ng/ml and 0.09±0.06 mg/ml (Fig. 5).

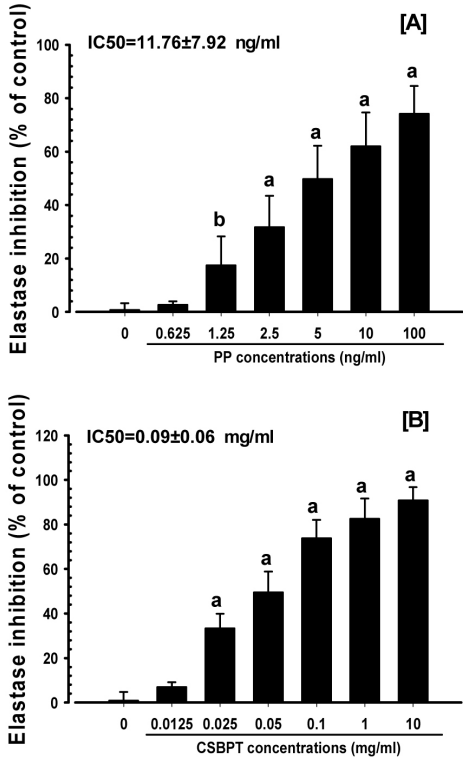

Fig. 5. Elastase inhibitory activity of PP [A] and CSBPT [B].
a : p<0.01 and b : p<0.05 compared with control by LSD test

4) Collagenase inhibitory activity

Collagenase inhibitory activity's attended (p<0.01 or p<0.05) increasing was detected from 0.025 mg/ml concentration of OA and CSBPT treated samples, and accordingly, IC50 of OA and CSBPT on the collagenase activity are detected as 0.09±0.05 and 0.28±0.16 mg/ml (Fig. 6).

5) MMP-1 inhibitory activity

MMP-1 inhibitory activity was attended (p<0.01) inhibited by treatment of OA and CSBPT from 0.025 mg/ml concentrations, and accordingly, IC50 of OA and CSBPT on the MMP-1 activity are detected as 0.39±0.10 and 0.59±0.34 mg/ml (Fig. 7).

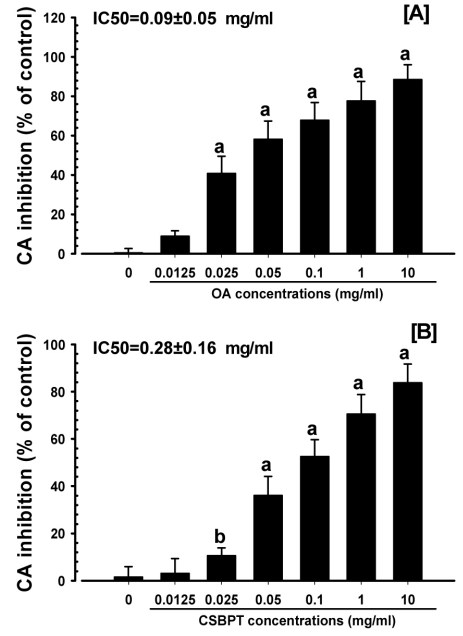

Fig. 6. Collagenase inhibitory activity of OA [A] and CSBPT [B].
a : p<0.01 and b : p<0.05 compared with control by LSD test

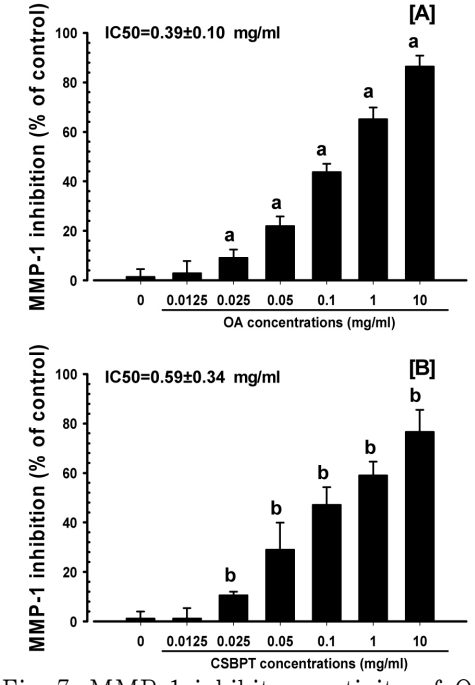

Fig. 7. MMP-1 inhibitory activity of OA [A] and CSBPT [B].
a : p<0.01 compared with control by LSD test
b : p<0.01 compared with control by MW test

3. Whitening effects

1) Tyrosinase inhibitory activity

Arbutin and CSBPT attended ($p<0.01$) inhibited mushroom tyrosinase activities from 20 and 25 μg/ml concentrations, and accordingly, IC50 of arbutin and CSBPT on the tyrosinase activity are detected as 71.09±11.13 and 66.14±18.82 μg/ml (Fig. 8).

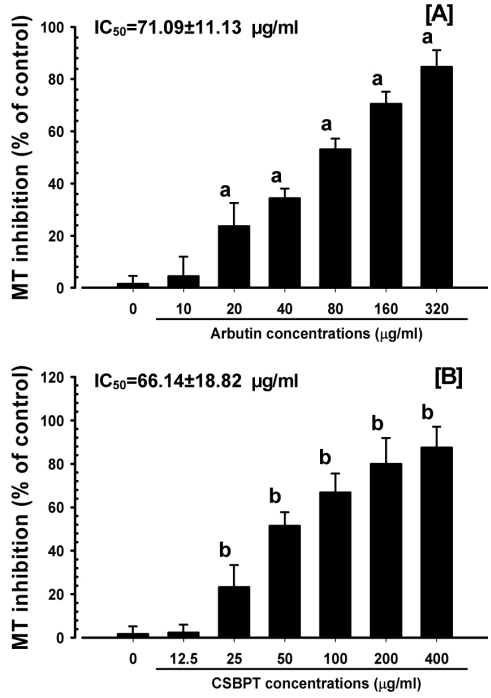

Fig. 8. MT inhibitory activity of Arbutin [A] and CSBPT [B].
a : $p<0.01$ compared with control by LSD test
b : $p<0.01$ compared with control by MW test

2) Melanin formation inhibitory activity

Arbutin and CSBPT also attended ($p<0.01$ or $p<0.05$) inhibited the B16/F10 melanoma cell melanin productions from 80 and 50 μg/ml concentrations, and accordingly, IC50 of arbutin and CSBPT on the melanin production are detected as 173.61±66.78 and 316.35±94.11 μg/ml (Fig. 9).

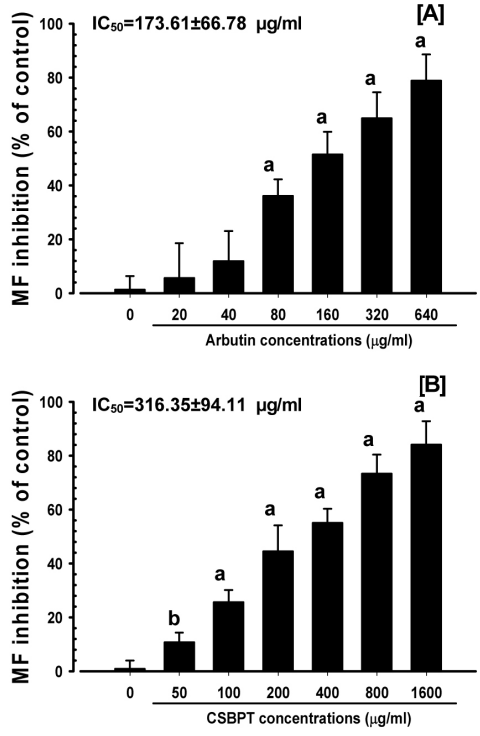

Fig. 9. MF inhibitory activity of Arbutin [A] and CSBPT [B].
a : $p<0.01$ and b : $p<0.05$ compared with control by LSD test

4. In vivo skin moisturizing effects

1) Changes on the body weights

No attended changing on the body weight and obtainment during 7 days of experiment periods were detected in all CSBPT treated mice as compared with vehicle control mice (Table 2, Fig. 10).

The body weight obtains during 7 days of experimental periods in CSBPT 500, 250 and 125 mg/kg administered mice showed 6.59, -2.20 and 3.30% point changes compared to vehicle control mice.

Table 2. Changes on the Body Weight Obtainments on the in vivo Mouse Skin Moisturizing Assay

Times groups	Body weights at		
	First administration* [A]	Sacrifice* [B]	Weight gains [B-A]
Controls			
Intact	29.13	30.26	1.14
vehicle	±2.46	±2.26	±0.67
CSBPT treated			
500	29.25	30.46	1.21
mg/kg	±2.58	±2.52	±0.39
250	29.36	30.48	1.11
mg/kg	±2.32	±2.36	±0.24
125	29.34	30.51	1.18
mg/kg	±2.28	±2.15	±0.47

Values are expressed as Mean±S.D. of eight mice, g.
*All animals were overnight fasted.

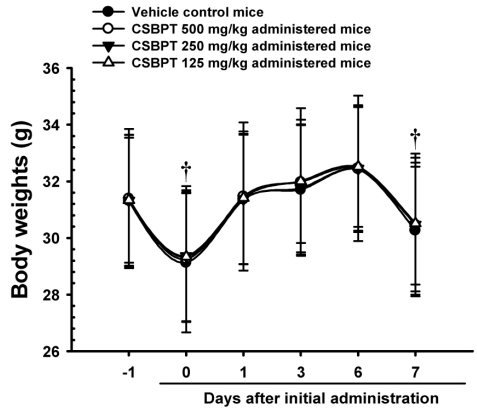

Fig. 10. Body weight changes on the in vivo mouse skin moisturizing assay.
All animals were fasted overnight before the first administration and sacrifice, the day 7 (†).

2) Effects on skin water contents

Skin water contents's attended ($p<0.01$) increasing was detected by oral treatment of all 3 different dosages of CSBPT, 500, 250 and 125 mg/kg, dose-dependently compared to vehicle control mice at 24 hrs after 7 th administration(Fig. 11).

The skin water contents in CSBPT 500, 250 and 125 mg/kg administered mice showed 47.33, 40.06 and 31.34% point changes compared with vehicle control mice.

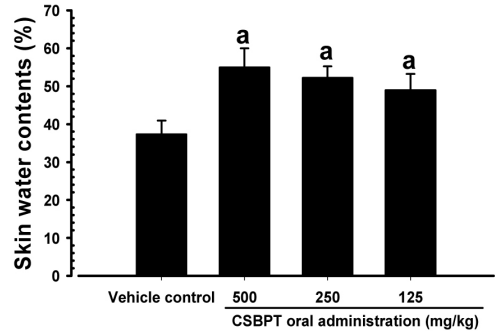

Fig. 11. Changes on the skin water contents on the in vivo mouse skin moisturizing assay.
a : $p<0.01$ compared with whole vehicle control by LSD test

Ⅳ. Discussion

Skin aging is a change in the skin that occur due to natural aging and photoaging. The clinical symptoms of skin aging is irregular dryness, dark/light pigmentation, deep furrows or severe atrophy, sallowness, dehydrogenation, telangiectases, premalignant lesions, laxity, and leathery appearance[19]. Skin aging in Korean Medicine has been recognized as a process of natural aging. But in modern life it is extended and improved the quality of life. In the high demands of the public who want to spend a healthy old age. The skin is exposed where the process of aging. So

there is also increasing concern that you want to have a bright and elastic skin and investment. The active research has been done on the skin change due to aging in the medical community[20].

In Korean Medicine, references about the aging process can be found ≪Somun · Sanggocheonjinnon≫. Describing the structural and functional changes associated with aging, and it have expressed changes in skin and hair with age. It also mentioned skin as a basis for understanding the process of aging[21].

In Korean Medicine, wrinkles caused by aging of the dermis and subcutaneous fat have been thought the phenomenon occurred by a reduction of spleen function. In the case of pigmentation, unfair and excessive thinking occurs phlegm and it has been thought the cause of pigmentation. In addition, the deficiencies in pulmonary function can not enrich the skin moisture. It is organized by the overall aging of the skin in the deterioration of renal function[22].

Cheongsangbangping-tang is a mixed herbal formula by consisting of 12 herbs, and has been mainly used for healing facial comedones in Korean Medicine[3,4]. Favorable effects of *Cheongsangbangping-tang* on acne have been reported[3,4] with favorable effects on atopic dermatitis in rats[23]. And especially *Cheongsangbangping-tang* was rated as having antioxidant[5].

Skin aging has been associated with various and complex cells, extracellular factors. Particularly, the reactive oxygen species (ROS)[24], degradation of extracellular matrix (ECM)[25] and melanin pigmentations[26], decrease of moisture in keratin layers[27,28] have attracted attention. Anti-aging treatments research has also focused on antioxidant, anti-wrinkle, whitening, moisturizing the skin[13,29].

In the present study, therefore, we intend to observe the possible cytotoxicity, skin-regeneration, whitening, anti-wrinkle and skin moisturizing effects of CSBPT which is compared with those of well-documented standard references, TGF-β 1 in collagen type I synthesis, OA in hyaluronidase, collagenase and MMP-1 inhibitory assays, PP in elastase inhibitory effects, and arbutin in tyrosinase inhibitory assay and melanin formation test.

Cytotoxicity of CSBPT was experimented with human normal fibroblast cells, B16F10 murine melanoma cells by MTT assay. Skin regeneration and anti-wrinkle effects were also experimented with collagen type I synthesis assay by an EIA kit as comparing with TGF-β1, hyaluronidase, collagenase and MMP-1 inhibitory assays as comparing with OA, and elastase inhibitory effects as comparing with PP. In addition, whitening effects of CSBPT were additionally observed by tyrosinase inhibitory assay and melanin formation test in B16/F10 melanoma cells as comparing with arbutin, and skin moisturizing effects were measured by mouse skin water contents test. In in vivo skin moisturizing effects, eight mice in each group were ready, and the

contents of water (%) in 2×3 cm of dorsal hair clipped back skin were calculated by automated moisture analyzers balance at 24 hrs after last 7 th oral administration of vehicle, CSBPT 500, 250 and 125 mg/kg, in this experiment.

As results of this experiment, no CSBPT treatment related cytotoxic effects were demonstrated against human normal fibroblast, B16/F10 murine melanoma cells from 1.25 to 500 mg/ml concentrations, the lowest to the highest concentrations tested in this experiment. CSBPT concentration-dependent increased collagen type I synthesis at human normal fibroblast cells. It was also supressed hyaluronidase, elastase, collagenase and MMP-1 activities, enzymes that are continuous with degradation of ECM and formation of wrinkle[6,10,13]. In addition, CSBPT supressed melanin productions of B16/F10 melanoma cells compared with tyrosinase activity, an enzyme connected with melanin formation[30,31], and dose-dependent and significant increases of skin water contents were detected in CSBPT treated mouse skin as compared with vehicle control skins, in this experiment. These are considered as direct evidences that CSBPT showed favorable and enough skin regeneration, anti-wrinkle, whitening and skin moisturizing effects as serve as a predictable functional ingredient for skin aging. Standard references - Arbutin[32], TGF-β[16,33], PP[34,35] or OA[13,36] also showed favorable anti-wrinkle, skin regeneration or whitening effects, similar to those known in the literature and results of the present study are judged that there is no error.

Arbutin is a glycosylated hydroquinone extracted from the bearberry plant in the genus Arctostaphylos. It inhibits tyrosinase. So it protects melanin formation. Arbutin is therefore, have been used as a skin-lightening agent[32,37,38]. TGF-β1 is a polypeptide member of the TGF-β superfamily of cytokines. It is a secreted protein that carry out many cellular functions, including the control of cell proliferation, cell growth, cell differentiation and apoptosis[39,40], and also critical roles on the skin collagen synthesis[6,33]. Phosphoramidon disodium salt (PP) is a chemical compound derived from cultures of Streptomyces tanashiensis, and is an inhibitor of the various enzymes including elastase[34,35,41]. Oleanolic acid (OA), which is a pentacyclic triterpene found in several plant extract to have chemopreventive, anti-inflammatory, skin protective property[25,42,43]. It has been well known that botanical extracts containing OA are useful in skin care functional foods[13]. OA has regenerative properties in case of sun damaged skin. Local application of this pentacyclic triterpene, used by anti-wrinkle ingredient, restore the damaged collagen fiber bundle[36]. These evidences suggested us to select arbutin, TGF-β1, OA and PP as standard reference compound for, skin-regeneration, anti-wrinkle or whitening effects, in this experiment.

Type I collagen is the main structural protein in the skin. Collagen destruction is thought to the root cause of aging skin and changes outcoming from chronic sun exposure[33]. Skin wrinkles are a complex process of abortion that involves declining age-related skin cell function. It causes harmful proteolysis of ECM which leaves visible signs on the surface[25]. Some scientific evidences for skin wrinkle emphasizes the degradation of ECM, which is involved in increased skin enzymatic activity and wrinkling , such as hyaluronidase, collagenase, elastase and MMP-1[10,25]. Skin-care ingredients have the potential to synthesis of type I collagen and/or inhibit the activity of enzymes that involved in degradation of ECM in aged skin, can be used as a wrinkle remover[6,19]. In this experiment, TGF-β1 used by standard collagen synthesis reference came out potent increases of type I pro-collagen synthesis (EC50 = 18.93±8.72 ng/ml), PP used as elastase inhibitory standard reference showed potent inhibitory effects to elastase (IC50 = 11.76±7.92 ng/ml), also OA used as hyaluronidase, collagenase and MMP-1 inhibitory standard reference showed potent inhibitory effects to hyaluronidase (IC50 = 0.11±0.09 mg/ml), collagenase (IC50 = 0.09±0.05 mg/ml) and MMP-1 (IC50 = 0.39±0.10 mg/ml), respectively. In addition, CSBPT also showed concentration dependent increasing of type I collagen synthesis of fibroblasts (EC50 = 0.10±0.06 mg/ml) without cytotoxicity against human normal fibroblast cells, up to 500 mg/ml concentrations, the highest concentration tested in this experiment, and also showed favorable inhibitions to hyaluronidase (IC50 = 0.13±0.06 mg/ml), elastase (IC50 = 0.09±0.06 mg/ml), collagenase (IC50 = 0.28±0.16 mg/ml) and MMP-1 (IC50 = 0.59±0.34 mg/ml), as direct evidenced that CSBPT has adequate anti-wrinkle effects without cytotoxicity, and expected as an ingredient for functional ingredients for skin aging.

Melanin is the main component that determines the color of skin. Up to 10% of cells in the far ben layer of the epidermis produce melanin pigments[44]. The main part of melanin is protecting the skin from ultraviolet skin damage[45]. Melanin biosynthesis or melanin production is a well-known physiological response to human skin when exposed to ultraviolet light and other stimuli. Melanin production is regulated by enzymes like tyrosinase, tyrosinase related protein (TRP)-1 and TRP-2[46]. The inhibition of tyrosinase is the most common method of achieving skin whitening because it is the important enzyme that promotes the rate limiting step of melanin biosynthesis[26,47]. And the murine B16/F10 cell line was used because of producing melanin, which are known containing tyrosinase which is associated with melanine production, respond to α-MSH vitalozation and easy to incubate in vitro[48,49]. According to our study, arbutin was used as standard whitening reference and showed strong

inhibitory effects on mushroom tyrosinase (IC50 = 71.09±11.13 μg/ml) and melanin produces of B16/F10 melanoma cells (IC50 = 173.61±66.78 μg/ml). In addition, CSBPT also showed concentration dependent inhibitions to tyrosinase (IC50 = 66.14± 18.82 μg/ml) and melanin produces of B16/F10 melanoma cells (IC50 = 316.35 ±94.11 μg/ml) without cytotoxicity, up to 500 mg/ml concentrations, as direct evidenced that CSBPT has adequate whitening effects.

A normal human keratin layer maintains moisture in the skin. they contain 10-20% of waters, but decreasing the water content of keratin layers accelerates the aging process, such as wrinkles and itching[28]. Because of this fact, keratin layers should contain enough moisture to maintain healthy, resilient skin even in dry conditions[50]. The skin moisturizing effect can be easily detected by moisture contents through animal skins or human pilot study[13-5]. Attended ($p<0.01$) and dose-dependent increases of skin water contents were detected in CSBPT 500, 250 and 125 mg/kg administered mouse dorsal back skins compared with vehicle control mice at 24 hrs after last 7 th treatment, suggesting CSBPT has a strong skin moisturizing effect and can be expected as a powerful ingredient in the present study. No attended changing the body weight were detected in all three different dosages of CSBPT administered mice, 500, 250 and 125 mg/kg as compared with vehicle control mice; consequently, administration of CSBPT did not affected the body weight gains during 7 days of continues oral administration periods in the current result.

Taken together the results of this experiment, it is suggested that CSBPT showed favorable and enough skin regeneration, anti-wrinkle, whitening and skin moisturizing effects as serve as a predictable functional ingredient for skin aging, at least in a condition of this study. In addition, standard references used in this study - arbutin, TGF-β1 OA, or PP also found to be favorable skin regeneration, anti-wrinkle or whitening effects, within reference values, the experiment protocol and results of this experiment are acceptable. However, more detail mechanism and in vivo skin protective efficacy studies should be conducted in future with the screening of the biological active compounds in individual herbs of CSBPT.

V. Conclusion

In this experiment, we concluded the following results:

1. CSBPT did not have cytotoxic effects on human normal fibroblast cells, B16/F10 murine melanoma cells.
2. The type I collagen synthesis was increased on TGF-β1 and CSBPT-treated fibroblast cells.
3. Hyaluronidase activities were inhibited

in OA and CSBPT-treated samples that were concentration dependent.

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

According to these results, CSBPT is expected to act as a functional ingredient that prevents and slows down skin aging.

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국문초록

목 적: 본 연구는 한의학에서 면포질환에 빈번히 사용하는 대표적인 복합처방인 청상방풍탕의 열수 추출물 동결건조물의 피부 노화 개선 효과 평가를 위하여 피부재생, 주름개선, 미백 및 보습 효과를 각각 평가하였고, 기본적인 독성 평가의 일환으로 세포독성 또한 in vitro 방법으로 평가하였다.

방 법: 본 연구에서는 human normal fibroblast(CRL-2076) 세포 및 B16/F10 murine melanoma(CRL-6475) 세포에 대한 청상방풍탕 열수 추출 동결건조물(수율 18.71%)의 세포독성을 MTT(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium Bromide) 방법을 통해 평가하였고, B16/F10 melanoma cells의 melanin 생성 억제 정도 및 melanin 합성 필수 효소인 tyrosinase 활성 억제와 arbutin을 비교 평가하여 미백효과를 평가하였다. 또한 피부 재생 및 주름 개선 효과를 transforming growth factor(TGF)-β1와 fibroblast의 collagen type I 합성능을 비교, phosphoramidon disodium salt(PP)와 세포외 기질의 분해에 관여하는 elastase활성 억제를 비교, oleanolic acid(OA)와 hyaluronidase, collagenase 및 matrix metalloproteinase(MMP)-1 활성 억제를 비교하여 각각 평가하였고, 마우스 피부 수분함량 변화를 관찰하여 보습효과를 평가하였다.

결 과: 본 실험의 결과, 청상방풍탕은 human normal fibroblast 세포 및 B16/F10 murine melanoma 세포에서 최고 농도인 500 mg/ml까지 유의한 세포독성이 나타나지 않았고, fibroblast의 collagen type I 합성을 증가시켰다. 또한 세포외기질 파괴와 연관된 것으로 알려진 hyaluronidase, elastase, collagenase 및 MMP-1 활성을 각각 억제하였고 melanin의 생성에 관여하는 tyrosinase의 활성 및 B16/F10 melanoma 세포의 melanin 생성을 농도 의존적으로 차단함이 관찰되었다. 이와 더불어 정상매체 대조군에 비해 청상방풍탕 경구 투여군이 투여 용량 의존적으로 마우스 피부 수분 함량을 유의성 있게 증가시켰다.

결 론: 이상의 결과에서, 청상방풍탕은 세포 독성 없이 피부재생, 주름개선, 미백 및 보습 효과를 나타남이 관찰되어 차후 피부 개선 소재로서 그 가치가 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 vivo평가를 체계적으로 수행해야 할 것으로 판단된다.

중심단어: 청상방풍탕, 피부재생, 항노화, 미백, 보습

Reference

1. Kim HC, Oh KD. Review in the Effects Influencing Skin Aging and Its Prevention and Cure. Asian J of Beauty and Cosmetology. 2011;9(1):1-9.
2. Jung MS. Study on Middle-Aged Women's Understanding of Facial Skin Aging and Desire to Improve. Master thesis. Seokyung Univ. 2014.
3. Rhee SH. The clinical classification as identify of symptoms about 54 cases of patients with Acne. Korean J Korean Medical Institute of Dermatology and Aesthetics. 2005;1(1):114-26.
4. Kim JH, Yoo DY. A case report of acne vulgaris patient treated with

Cheongsangbangpung-tang. The J of Daejeon Korean Medicine. 2013;22(1):185-91.
5. Seo EJ, Hwang CY, Kim NK. Experimental study of *GagamCheongsang BangPung-Tang* on the anti-inflammatory effects. The J of Korean Med Ophthalmology & Otolaryngology & Dermatology. 2008;21(2):54-70.
6. Kim YH, et al. Anti-wrinkle activity of ziyuglycoside I isolated from a *Sanguisorba officinalis* root extract and its application as a cosmeceutical ingredient. Biosci Biotechnol Biochem. 2008;72(2):303-11.
7. Nema NK, et al. *Cucumis sativus* fruit-potential antioxidant, anti-hyaluronidase, and anti-elastase agent. Arch Dermatol Res. 2011;303(4):247-52.
8. Heo JC, et al. Anti-oxidant and anti-tumor activities of crude extracts by *Gastrodia elata* blume. Korean J Food Presev. 2006;13(1):83-7.
9. Wünsch E, Heindrich HG. Zur quantitativen bestimmung der kollagenase. Fresenius' J Analy Chem. 1964;203:397-8.
10. Losso JN, et al. Inhibition of matrix metalloproteinase-1 activity by the soybean Bowman-Birk inhibitor. Biotechnol Lett. 2004;26(11):901-5.
11. Masamoto Y, et al. Mushroom tyrosinase inhibitory activity of esculetin isolated from seeds of Euphorbia lathyris L. Biosci Biotechnol Biochem 2003;67(3):631-4.
12. Hosoi J, et al. Regulation of melanin synthesis of B16 mouse melanoma cells by 1 alpha, 25-dihydroxyvitamin D3 and retinoic acid. Cancer Res. 1985;45(4):1474-8.
13. Kim KH, et al. Anti-skin-aging benefits of exopolymers from *Aureobasidium pullulans* SM-2001. J Cosmet Sci. 2014;65(5):285-98.
14. Camargo FB Jr, Gaspar LR, Maia Campos PM. Skin moisturizing effects of panthenol-based formulations. J Cosmet Sci. 2011;62(4):361-70.
15. Kanlayavattanakul M, Rodchuea C, Lourith N. Moisturizing effect of alcohol-based hand rub containing okra polysaccharide. Int J Cosmet Sci. 2012;34(3):280-3.
16. Kang SJ, et al. Fermentation with *Aquilariae Lignum* enhances the anti-diabetic activity of green tea in type II diabetic db/db mouse. Nutrients. 2014;6(9):3536-71.
17. Levene A. Pathological factors influencing excision of tumours in the head and neck. Part I. Clin Otolaryngol Allied Sci. 1981;6(2):145-51.
18. Ludbrook J. Update: microcomputer statistics packages. A personal view. Clin Exp Pharmacol Physiol. 1997;24(3-4):294-6.
19. Gilchrest BA. Skin aging and photoaging. Dermatol Nurs. 1990;2(2):79-82.
20. Seong EJ, et al. Treatment of Wrinkles with Oriental Medicine. The J of Korean Med Ophthalmology & Otolaryngology & Dermatology. 2008;21(3):184-99.
21. Jung HJ. A study on the Sanggocheunron of the Somoon. Master thesis. Wonkwang

Univ. 2000.
22. Han JM et al. The Study on the Korean and Western Medical Literatures for Skin Aging wrinkle, hyperpigmentation, dry skin, facial flush. The J of Korean Med Ophthalmology & Otolaryngology & Dermatology. 2014;21(2):1-13.
23. Jin KS, et al. Regulatory effect of atopic allergic reaction by *Chungsangbangpoong-tang*. The J of Korean Medicine Pediatrics. 2004;18(2):61-76.
24. Wiedow O, et al. Elafin: an elastase-specific inhibitor of human skin. Purification, characterization, and complete amino acid sequence. J Biol Chem. 1990;265(25):14791-5.
25. Maity N, et al. Exploring *Tagetes erecta* Linn flower for the elastase, hyaluronidase and MMP-1 inhibitory activity. J Ethnopharmacol. 2011;137(3):1300-5.
26. Chan YY, Kim KH, Cheah SH. Inhibitory effects of *Sargassum polycystum* on tyrosinase activity and melanin formation in B16F10 murine melanoma cells. J Ethnopharmacol. 2011;137(3):1183-8.
27. Imodawa G. Recent advances in characterizing biological mechanisms underlying UV-induced wrinkles: a pivotal role of fibroblast-derived elastase. Arch dermatol Res. 2008;300(Suppl 1):7-20.
28. Kim EJ, et al. Skin aging and photoaging alter fatty acids composition, including 11,14,17-eicosatrienoic acid, in the epidermis of human skin. The J of Korean Med Sci. 2010;25(6):980-3.
29. Cho EA, et al. Screening of anti-wrinkle resource from herbal medicinal extracts and stability test of its cosmetic products. Korean J Medicinal Crop Sci. 2011;19(2):126-35.
30. Parvez S, et al. Natural occurring tyrosinase inhibitors: mechanism and applications in skin health, cosmetic and agriculture industries. Phytother Res. 2007;21(9):805-16.
31. Parvez S, et al. Survey and mechanism of skin depigmenting and lightening agents. Phytother Res. 2006;20(11):921-34.
32. Jung HK, et al. Whitening and anti-wrinkle effects of Korean native *Dendrobium moniliforme* methanol extract. Korean J Medicinal Crop Sci. 2014;22(5):331-8.
33. Liao PL, et al. Anti-ageing effects of alpha-naphthoflavone on normal and UVB-irradiated human skin fibroblasts. Exp Dermatol. 2012;21(7):546-8.
34. Kawaharajo K, et al. Effect of phosphoramidon on protection against corneal ulcer caused by elastase and protease from *Pseudomonas aeruginosa*. Jpn J Exp Med. 1982;52(5):271-2.
35. Bernard E, Hornebeck W, Robert L. Elastase-type endopeptidase of fibroblasts. Effect of metalloprotease inhibitors. C R Acad Sci III. 1995;318(2):179-82.
36. Nishimori Y, et al. UVB-induced ultrastructural changes of collagen bundles in hairless mouse skin. J Dermatol Sci. 1997;15:125.

37. O'Donoghue JL. Hydroquinone and its analogues in dermatology - a risk-benefit viewpoint. J Cosmet Dermatol. 2006;5(3):196-203.
38. Pop C, Vlase L, Tamas M. Natural resources containing arbutin. Determination of arbutin in the leaves of *Bergenia crassifolia* (L.) Fritsch. acclimated in Romania. Not Bot Hort Agrobot Cluj. 2009;37(1):129-32.
39. Assoian RK, et al. Transforming growth factor-beta in human platelets. Identification of a major storage site, purification, and characterization. J Biol Chem. 1983;258(11):7155-60.
40. Jablonska E, et al. Transforming growth factor-β1 induces expression of human coagulation factor XII via Smad3 and JNK signaling pathways in human lung fibroblasts. J Biol Chem. 2010;285(15):11638-51.
41. Kitagishi K, Hiromi K. Binding between thermolysin and its specific inhibitor, phosphoramidon. J Biochem. 1984;95(2):529-34.
42. Aguirre MC, et al. Topical anti-inflammatory activity of 2alpha-hydroxy pentacyclic triterpene acids from the leaves of *Ugni molinae*. Bioorg Med Chem. 2006;14(16):5673-7.
43. Juan ME, et al. Antiproliferative and apoptosis-inducing effects of maslinic and oleanolic acids, two pentacyclic triterpenes from olives, on HT-29 colon cancer cells. Br J Nutr. 2008;100(1):36-43.
44. Hearing VJ. Biogenesis of pigment granules: a sensitive way to regulate melanocyte function. J Dermatol Sci. 2005;37(1):3-14.
45. Tsatmali M, Ancans J, Thody AJ. Melanocyte function and its control by melanocortin peptides. J Histochem Cytochem. 2002;50(2):125-33.
46. Kameyama K, et al. The expression of tyrosinase, tyrosinase-related proteins 1 and 2 (TRP1 and TRP2), the silver protein, and a melanogenic inhibitor in human melanoma cells of differing melanogenic activities. Pigment Cell Res. 1995;8(2):97-104.
47. Solano F, et al. Hypopigmenting agents: an updated review on biological, chemical and clinical aspects. Pigment Cell Res. 2006;19(6):550-71.
48. An BJ, et al. Antioxidant effects and application as natural ingredients of Korean *Sanguisorbae officinalis* L. J Korean Soc Appl Biol Chem. 2004;47(2):244-50.
49. Buscà R, Ballotti R. Cyclic AMP a key messenger in the regulation of skin pigmentation. Pigment Cell Res. 2000;13(2):60-9.
50. Jacobi O. About the mechanism of moisture regulation in the horny layer of the skin. Proc Sci Sec Toilet Goods Assoc. 1959;31:22.