대한예방한의학회지 제22권 제2호(2018년 8월) doi : 10.25153/spkom.2018.22.2.009
Journal of Society of Preventive Korean Medicine 2018 ; 22(2) : 109-123

In vitro anti-skin-aging effects of dried pomegranate concentrated powder

Dae-Geon Lee[1),2)#], Beom-Rak Choi[3),4)#], Sae-Kwang Ku[1),2)], Su-Jin Kang[1),5)],
Hye-Rim Park[3)], Mi-Sun Sung[3)], Young-Joon Lee[1),5)*] & Ki-Moon Park[4)*]

[1)] The Medical Research Center for Globalization of Herbal Medicine, Daegu Haany University, Gyeongsan Gyeongsangbuk-Do
[2)] Department of Histology and Anatomy, College of Korean Medicine, Daegu Haany University, Gyeongsan Gyeongsangbuk-Do
[3)] Research Institute, HL Science Co., Ltd., Anyang
[4)] Department of Foodscience and biotechnology, Sungkyunkwan University, Suwon
[5)] Department of Preventive Medicine, College of Korean Medicine, Daegu Haany University, Gyeongsan Gyeongsangbuk-Do

Abstract

Purpose : In this study, we intended to observe the anti-wrinkle and moisturizing effects of dried pomegranate juice concentration powder (PCP) using *in vitro* test.

Materials and methods : Antioxidant effects of PCP were determined by free radical scavenging capacity (DPPH assay) and the cytotoxicity of PCP was examined in human keratinocyte (HaCaT) and human primary dermal fibroblast-neonatal (HDF) cells. To investigate the moisturizing effect of PCP, hyaluronan synthesis was examined in HaCaT cells. Activity of procollagen production were assessed in HDF cells and elastase inhibition properties of PCP were evaluated in cell free condition, to determine their anti-wrinkle effects. Metalloproteinase 1 (MMP-1) activity was also assessed following UVB irradiation, in the current *in vitro* experiment.

Results : No PCP treatment related significant cytotoxic effects were demonstrated against to the both HDF and HaCaT cells. PCP showed favorable free radical scavenging activities in dose-dependent manner. In PCP-treated HaCaT cells, hyaluronan synthesis was non-significantly but markedly increased, and pro-collagen productions were significantly increased in HDF cells, at all three different concentrations (0.25, 0.75 and 1 mg/ml), and elastase inhibitory activities were observed by PCP treatment. A significant decrease in UVB-induced MMP-1 activity was also observed in 1 mg/ml PCP-treated HDF cells as compared to those of UVB-exposed cells.

Conclusions : Taken together, these results suggest that PCP has favorable antioxidant, anti-wrinkle and moisturizing effects without meaningful cytotoxicity on HDF and HaCaT cell lines.

Key words : Dried pomegranate juice concentration powder, Antioxidant, Anti-wrinkle, Moisturizing effects, *In vitro*

• 접수 : 2018년 7월 9일 • 수정접수 : 2018년 7월 19일 • 채택 : 2018년 7월 22일

*교신저자 : Young-Joon Lee, Department of Preventive medicine, College of Korean Medicine, Daegu Haany University, 1 Haanydaero, Gyeongsan, Gyeongsangbuk-Do, 38610, Republic of Korea
전화 : +82-53-819-1296, 팩스 : +82-53-819-1576, 전자우편 : gksxntk@dhu.ac.kr
Ki-Moon Park, Department of Foodscience and biotechnology, Sungkyunkwan University, 2066 Seoburo, Jangan-gu, Suwon, Gyeonggi-do, 16419, Republic of Korea.
전화 : +82-31-290-7806, 전자우편 : E-mail : Pkm1001@skku.edu
# These authors contributed equally to this work.

I. Introduction

Skin aging is characterized by clinical signs including wrinkles, irregular dryness, dyspigmentation, sallowness, deep furrows or severe atrophy, dehydration, telangiectases, premalignant lesions, laxity and a leathery appearance of skin[1]. Skin aging is a complex biological process involving intrinsic factors (genetic factors, hormonal status and metabolic reactions, such as oxidative stress) and extrinsic factors (chronic light exposure, pollution, ionizing radiation, chemicals and toxins). A combination of these factors causes physiological alterations and progressive changes in each skin layer, and concomitant changes in skin appearance[2]. Oxidative stress caused by reactive oxygen species (ROS) plays a pivotal role in the process of skin aging at the cellular level[3,4]. ROS can block the formation of collagen, disrupt cellular renewal cycles, damage DNA and stimulate the release of pro-inflammatory mediators (cytokines), which cause inflammatory skin diseases[5-8]. Additionally, ROS cause the depletion of antioxidant enzymes and destroy the cytoprotective defense mechanism by weakening antioxidant systems, thus rendering the skin susceptible to oxidative injury[9-11]. Native human melanin consists of eumelanin and pheomelanin, and eumelanin is found in almost every type of human skin[12,13]. In the skin, melanin synthesized in melanocytes, which are located in the basal layer and hair bulbs, transfers to keratinocytes. Melanin in keratinocytes acts as a photoprotector through body coloration and scavenging reactive oxygen species such as superoxide anion and singlet oxygen[14-19]. Despite the photoprotective role of melanin, many cosmetics have been developed to prevent melanin formation in the skin because of aesthetic satisfaction by whitening ability. Of these, inhibitor of tyrosinase, which is a pivotal enzyme for melanin synthesis[20], has been used as a major ingredient of cosmetics[21-25]. Tyrosinase, an enzyme which contains dinuclear copper ions at the active site[26-28], catalyzes two distinct reactions of melanin synthesis[29], the hydroxylation of a monophenol and the conversion of an o-diphenol to the corresponding o-quinone, indicating that L-tyrosine is hydroxylated to L-DOPA, which is in turn converted to dopaquinone.

To prevent and reduce skin aging, many people have used functional cosmetics that have a potent skin protective pharmacological effect (anti-aging, whitening, anti-wrinkle, moisturizing and skin-protective effects)[30]. Currently, there are various available ingredients for functional cosmetics in the market. However, they have a number of limitations; they are too expensive and have side effects, and their exact pharmacological mechanisms are not fully understood[31]. Due to these factors, various investigations have continuously attempted to search for affordable and effective functional ingredients, with fewer side effects, especially from natural sources[30, 32].

Pomegranate is a rich source of crude fibers, pectin, sugars, and several tannins[33]. In addition, it has been reported that pomegranate contains some species of flavonoids and anthocyanidins in their seed oil and juice, and shows an antioxidant activity three times more potently than red wine and green tea extract[34, 35]. Furthermore, the chemopreventive and adjuvant therapeutic applications of pomegranate to human breast cancer have been warranted recently[36]. Owing to these significant biological activities, pomegranate juice is being increasingly popularized in world wide. Therefore, it also considered that pomegranate extracts will be showed favorable effects on the skin aging, because several scientific evidences on skin aging highlight the ROS[32], degradation of extracellular matrix (ECM)[37] and melanin pigmentations[38], decrease of moisture in keratin layers[39,40]. Especially, we recently

reported that pomegranate juice concentrated solution (PCS) made by HL Science Co. (Uiwang, Korea) showed favorable in vivo and in vitro skin protective effects including UVB-induced photoaging[41,42], and dried pomegranate juice concentrated powder (PCP) also made by HL Science Co. (Uiwang, Korea) showed favorable skin whitening effects, *in vitro*[43] and *in vivo* skin protective activities against UVB-induced photoaging[44] and skin moisturizing effects through collagen and hyaluronan synthesis[45,46].

In the present study, therefore, we intended to observe the anti-wrinkle (elastase and matrix metalloproteinase (MMP)-1 inhibition, procollagen contents) and moisturizing (hyaluronan production) effects of PCP in *in vitro* test using human primary dermal fibroblast-neonatal (HDF) and human keratinocyte (HaCaT) cells with possible cytotoxicity on HDF and HaCaT cells, as a process for developing the potent alternative anti-skin aging agent.

II. Materials and methods

1. Test materials and chemicals

PCP was provided by HL Science Co. Ltd. (Uiwang, Korea). All test materials were maintained at 4℃ until use. PCP contains 1.15 mg/g of Ellagic acid. The concentrations ranges of PCP were used from 0.25 to 8 mg/ml. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2,2-Diphenyl-1-picrylhydrazyl (DPPH), retinoic acid (RA), L-Ascorbic acid (Vit C), and (+)-α-Tocopherol (Vit E) were purchased from Sigma-Aldrich (St. Louise, MO, USA). TGF-β was obtained from R&D Systems (Minneapolis, MN, USA). Fibroblast basal medium (FBM), FBM low serum kit, and FBM serum free kit for HDF cell lines were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA).

2. Cell cultures

HaCaT cells were purchased from ATCC and cultured according to the manufacturer's protocols. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) with 10% fetal bovine serum (FBS; Lonza, Walkersville, MD, USA), 100 μg/mL streptomycin (Sigma-Aldrich, St. Louis, MO, USA), and 100 U/ml penicillin (Sigma-Aldrich, St. Louis, MO, USA). HDF cell lines were obtained from ATCC and were cultured in fibroblast basal medium (FBM; ATCC, Manassas, VA, USA, PCS-201-030) supplemented with FBM low serum kit (ATCC, Manassas, VA, USA, PCS-201-041) and were used for the treatment with FBM serum free kit (ATCC, Manassas, VA, USA, PCS-201-040). Cells were cultured at 37℃ in a fully humidified atmosphere of 5% CO_2 using a commercial CO_2 incubator (Model 311, Thermo Forma, Marietta, OH, USA) and were passaged approximately every other day.

3. Cell viability assay

HaCaT and HDF cells were plated at a density of 5×10^4 and 6×10^3 cells/well in a 96-well plates, respectively. The HaCaT and HDF cells were exposed to various concentrations of PCP (0.75, 1, 1.5, 2 and 4 mg/ml) for 48 hrs and then cell proliferation reagent MTT (Sigma-Aldrich, St. Louis, MO, USA) was added to each well and incubated at a incubator for 2 hrs. The absorbance of the wells at 450 nm was read using a microplate reader (Sunrise, TECAN, Männedorf, Switzerland). The results were expressed in terms of IC_{50} (the concentration reach the cell viability as 50% of control).

4. DPPH radical scavenging activity test

DPPH radical scavenging assay was performed according to the method of Blois et al.[47]. Briefly, 0.2 mM solution of DPPH (Sigma-Aldrich, St. Louis, MO, USA) in methanol was prepared and used immediately. Each sample solution was diluted with distilled water to final PCP concentrations of 0.25, 0.5, 1, 1.5, 2, 4 and 8 mg/ml or final Vit C and E (1 mg/ml). The optical density (OD) at 517 nm was measured after 10 min with a UV/Vis spectrophotometer (Optizen Pop, Mecasys, Daejeon, Korea). The free radical scavenging activity was calculated as follows: DPPH radical scavenging activity (%) = 100 − [(OD_s / OD_c)×100]. OD_s is the absorbance of sample at 517 nm and OD_c is the absorbance of the vehicle-treated control at 517 nm. The results are reported in terms of IC_{50} (the concentration needed to reduce 50% of DPPH). Vit C and E were used as positive controls.

5. Hyaluronan production assay

To determine the activity of hyaluronan synthesis, HaCaT cells were treated with 0.25, 0.5, and 1 mg/ml PCP or 20 mM N-Acetyl-D-glucosamine (NAG; Sigma-Aldrich, St. Louis, MO, USA) for 24 hrs, and then cells were trypsinized and counted for normalization. Hyaluronan concentration in the samples was quantified using an enzyme-linked hyaluronan-binding protein sandwich assay (Cat# DY3614, R&D Systems, Minneapolis, MN, USA) based on manufacturer's methods[48].

6. Procollagen contents test

HDF cell (ATCC, Manassas, VA, USA, PCS-201-010) was seeded in 24 well at 2×10^4 cells/well and then cultured under cell culture conditions for 24 hrs. After incubation, the medium was replaced with 50 μg/ml medium containing the various concentration of PCP (0.25, 0.75 and 1 mg/ml) or TGF-β (R&D Systems, Minneapolis, MN, USA) 10 ng/ml mixed with serum-free kit and the cells were cultured for 24 hrs. The procollagen contents in culture supernatant were measured using Procollagen type I-c-peptodie (PIP) ELASA kit (MK101, Takara Bio, Tokyo, Japan) according to manufacturer's manual. Results were normalized on total protein content of the supernatant.

7. Elastase inhibition assay

The elastase inhibition assay was performed by measuring the release of p-nitroaniline due to proteolysis of N-succinyl-(Ala)3-p-nitroanilide by human leucocyte elastase (Sigma-Aldrich, St. Louis, MO, USA)[49] in the presence or absence of PCP 0.5, 0.75 and 1 mg/ml or PPR (Sigma-Aldrich, St. Louis, MO, USA) 10 μM and Vit C (Sigma-Aldrich, St. Louis, MO, USA) 50 μM as a standard under exactly the same experimental conditions. The absorbance was measured at 410 nm with a 96-well microplate reader and the elastase inhibitory activity of each sample was calculated using equation as follows: Elastase inhibitory activity (%) = 100 − [(OD_s / OD_c)×100], where OD_s is the absorbance of the experimental sample at 410 nm and OD_c is the absorbance of the vehicle-treated control at 410 nm. The results are reported in terms of IC_{50} (the concentration at which the percentage inhibition of elastase activity was 50%).

8. MMP-1 inhibition test

The assay was carried out using a fluorescence microplate with modified methods of Losso et al.[50]. HDF cells were incubated with PCP (0.5, 0.75, and 1 mg/ml) or 1 μM of RA in the absence or presence of UVB (5 mJ/cm^2) for 2 min, and

then cultured for 24 hrs. The UVB source was a UV irradiation crosslinker (Model CL-1000, UVP, Upland, CA, USA). Total MMP-1 protein levels in cell culture supernatants, which includes pro, active and inhibitor bound MMP-1, were quantified by ELISA (Human Total MMP-1 DuoSet; DY901; R&D Systems, Minneapolis, MN, USA) according to manufacturer's instructions.

9. Statistical analyses

All *in vitro* data were expressed as means ± standard deviation (SD) in three, four or six independent experiments, respectively. Multiple comparison tests for different dose groups were conducted. Variance homogeneity was examined using the Levene test. If the Levene test indicates no significant deviations, the obtained data was analyzed by one way ANOVA test followed by Tukey's multi-comparison test to determine which pairs of group are significantly different. In case of significant deviations 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 with Bonferroni's correction was conducted to determine the specific pairs of group. IC_{50} 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).

III. Results

1. Cytotoxicity of PCP in HaCaT and HDF cells

To investigate the cytotoxic effects of PCP and define an appropriate concentration for subsequent cell base assays, we performed MTT assays on HaCaT and HDF cell lines. Cell survival percentages, viability were calculated to determine the cytotoxic effects of various concentrations of PCP in HDF and HaCaT cells. PCP concentrations that did not affect cell viability were selected. In HaCaT cells, slight decreases of cell viabilities were detected in PCP 2 and 4 mg/ml treated groups as compared to those of non-treated control, but no significant changes on cell viabilities were observed at concentrations ranging from 0.75 to 4 mg/ml, the lowest to the highest

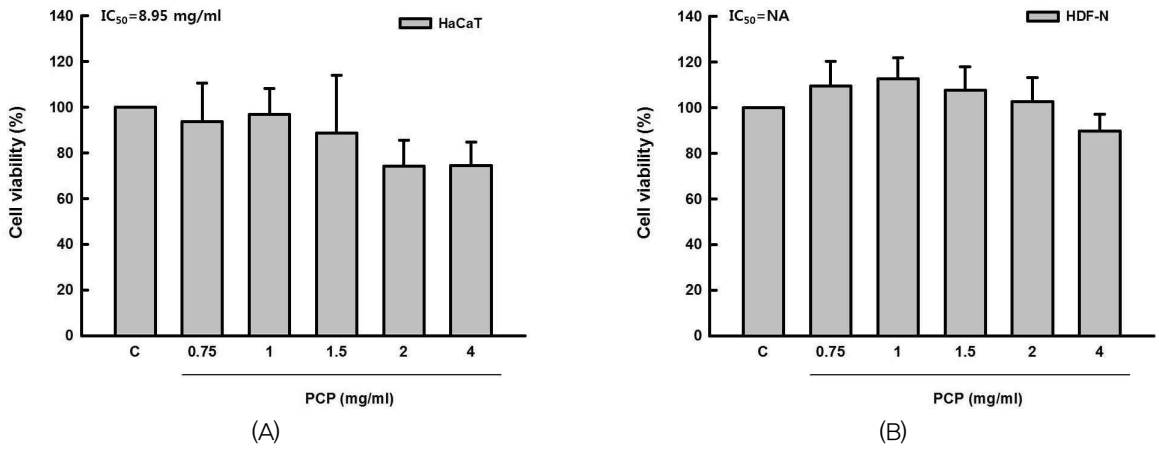

Figure 1. Cytotoxicity of PCP on HaCaT and HDF cells.

(A) HaCaT and (B) HDF cells were treated with various concentrations of PCP (0.75, 1, 1.5, 2, and 4 mg/ml) for 48 hrs. Values are expressed as means ± SD from six experiments. PCP = Pomegranate juice concentration powder; HDF = Human primary dermal fibroblast-neonatal cells; HaCaT = Human keratinocytes.

concentrations, tested in this MTT assay (Fig 1A). Although slight decreases of cell viabilities were detected in PCP 4 mg/ml treated groups as compared to those of non-treated control, no significant decreases of cell viabilities were also observed at concentrations ranging from 0.75 to 4 mg/ml, the lowest to the highest concentrations tested in this MTT assay, in HDF cells (Fig 1B). The IC50 values for the cytotoxicity of PCP were calculated as 8.95 mg/ml in HaCaT cells, but not applicable in HDF cells. The HaCaT cell viabilities were changed as 93.76±16.65, 96.75±11.48, 88.59±25.23, 74.12±11.35 and 74.42±10.36% in PCP 0.75, 1, 1.5, 2 and 4 mg/ml treated groups as compared to those of non-treated control, respectively. In addition, HDF cell viabilities were changed as 109.56±10.78, 112.70±9.05, 107.53±10.33, 102.68±10.56 and 89.66±7.34% in PCP 0.75, 1, 1.5, 2 and 4 mg/ml treated groups as compared to those of non-treated control, respectively.

2. Free radical scavenging activity of PCP

Significant increases in DPPH radical scavenging activities were detected in samples treated with Vit C (1 mg/ml) and E (1 mg/ml), and also in PCP at concentrations from 0.25 and 8 mg/ml, respectively. Vit C and E, and PCP significantly increased radical scavenging activity in a concentration dependent manner. In notice, PCP showed potent free radical scavenging activities as comparable to those of positive controls, Vit C and Vit E, at the concentration of over 1.5 mg/ml, in the current experiment (Fig 2). The IC_{50} values for the effect of PCP on anti-oxidation was calculated as 0.614 mg/ml. The DPPH radical scavenging activities were changed as 21.31±12.52, 38.48±2.06, 63.03±4.06, 84.54±2.97, 90.14±1.72, 89.47±1.46 and 87.06± 0.82% in PCP 0.25, 0.5, 1, 1.5, 2, 4 and 8 mg/ml treated samples, and as 91.83±1.41 and 91.67±1.43% in Vit A and C treated samples as compared to those of non-treated control, respectively.

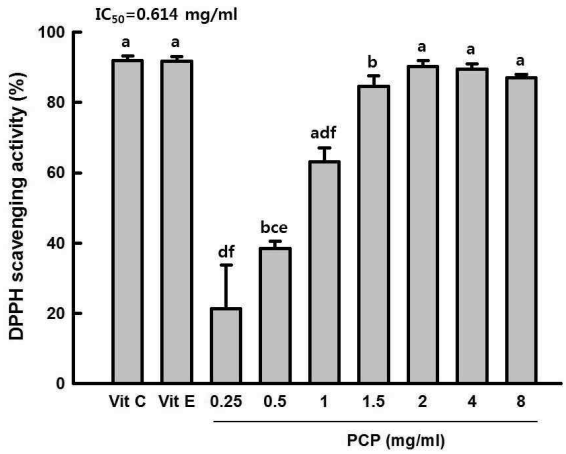

Figure 2. The antioxidant characteristics of PCP.
Values are expressed as means ± SD of three independent experiments. PCP = Pomegranate juice concen- tration powder; Vit C = Vitamin C; Vit E = Vitamin E; DPPH = 1-Diphenyl-2-picrylhydrazyl radical, 2,2-Diphenyl-1-(2,4,6-trinitrophenyl) hydrazyl. a $p<0.001$ and b $p<0.01$ as compared with control cells; c $p<0.001$ and d $p<0.05$ compared with Vit C treated cells; e $p<0.001$ and f $p<0.05$ compared with Vit E treated cells.

3. Moisturizing benefits of PCP

Moisturizing benefits were examined in PCP-treated HaCaT cells, which showed non-significant but markedly increase in hyaluronan synthesis following treatment with 0.25, 0.5 and 1 mg/ml PCP. As compared with control cells, the hyaluronan content of PCP-treated cells showed increases of 130.66±3.00, 139.48±19.49 and 128.69±21.47% on treatment with 0.25, 0.5 and 1 mg/ml PCP, respectively. In addition, 20 mM NAG significantly increased hyaluronan synthesis to 202.67±5.61% as compared with control cells (Fig 3).

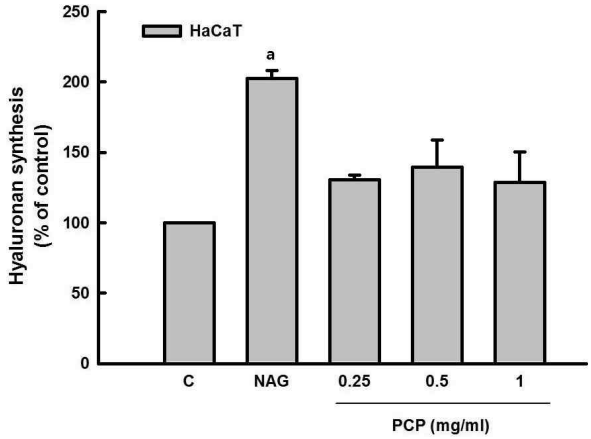

Figure 3. The effects of PCP on hyaluronan synthesis.

Values are expressed as means ± SD from three experiments. PCP = Pomegranate juice concentration powder; NAG=N-Acetyl-D-glucosamine; HaCaT = Human keratinocytes. a $p<0.05$ as compared with control cells.

4. Anti-wrinkle benefits of PCP

1) Procollagen synthesis: Procollagen synthesis was significantly increased by treatment of TGF-β and 0.5, 0.75, and 1 mg/ml PCP compared with control cells. TGF-β (10 ng/ml) altered the procollagen synthesis by 269.43±42.00%. In addition, procollagen synthesis was increased by 179.00±74.27, 139.10± 34.97 and 118.58±54.44% following treatment with 0.5, 0.75 and 1 mg/ml PCP, respectively (Fig 4).

2) Elastase inhibition: Significant inhibitions of elastase activity were observed in 1, 1.5 and 2 mg/ml PCP treatment as compared with vehicle treatment. Elastase inhibition activities were also increased by the addition of PPR (10 μM) and Vit C (50 μM), and also by PCP at 0.25, 0.5 and 4 mg/ml, but statistical significant was not observed in these treatment. PPR (10 μM) and Vit C (50 μ) decreased elastase activity by 39.03±13.13, 38.61±16.94 % compared with vehicle-treated cells. Elastase inhibition activity showed by 18.61±11.30, 24.46±10.65, 19.56±3.24, 13.34±3.03, 15.08± 3.14 and 6.05±5.36% following the addition of 0.25, 0.5, 1, 1.5, 2 and 4 mg/m PCP as compared with vehicle-treated cells, respectively (Fig 5).

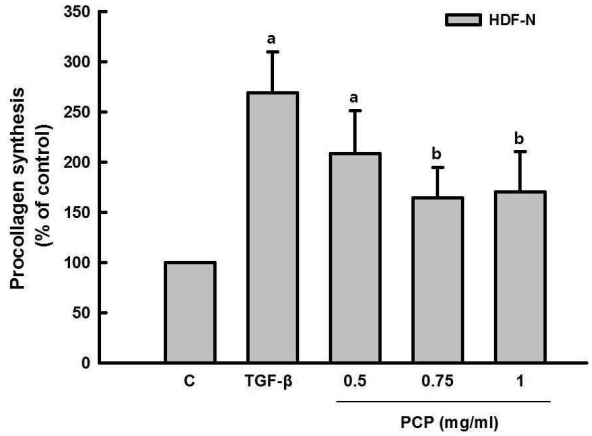

Figure 4. The effects of PCP on collagen synthesis.

Values are expressed as means ± SD from four experiments. PCP = Pomegranate juice concentration powder; TGF-β = Tumor growth factor-β; HDF= Human primary dermal fibroblast-neonatal cells. a $p<0.01$ and b $p<0.05$ as compared with control cells.

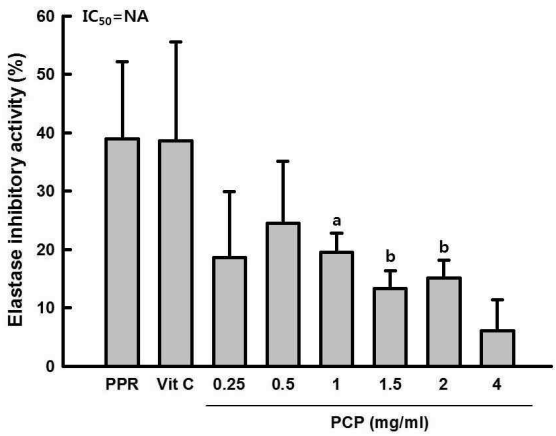

Figure 5. The effects of PCP on elastase inhibitory activity.

Values are expressed as the means ± SD from four independent experiments. PCP = Pomegranate juice concentration powder; PPR = Phosphoramidon; Vit C = Vitamin C. a $p<0.01$ and b $p<0.05$ as compared with control cells.

3) MMP-1 inhibition: UVB irradiation (50 mJ/cm^2) resulted in an increase as 149.82±11.63% in MMP-1 activity compared with control cells. However, UVB induced increase of MMP-1 activity was significantly reduced in 1 mg/ml PCP-treated cells. Compared with UV-exposed cells, MMP-1 activities were decreased by 21.24 ±15.85, 19.39±14.59 and 42.88±3.81% upon treatment with 0.5, 0.75, and 1 mg/ml PCP, respectively. In addition, RA 1 μM showed 9.35±8.86% decreases of MMP-1 activities as compared to UV-exposed cells, in the current analysis (Fig 6).

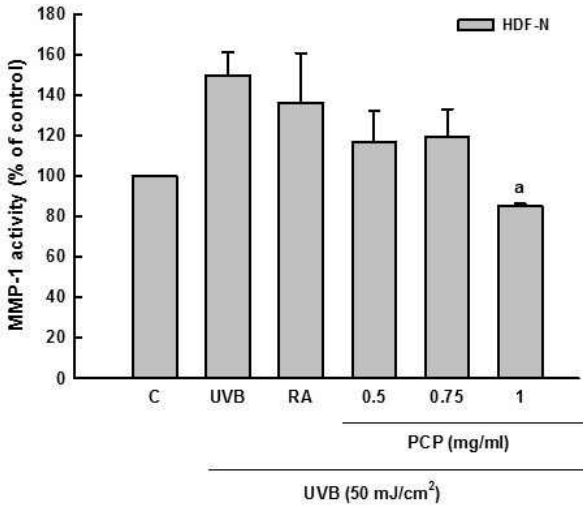

Figure 6. The effects of PCP on MMP-1 activity.

Values are expressed as means ± SD from three experiments. PCP = Pomegranate juice concentration powder; MMP-1 = Matrix metalloprotease 1; UVB = Ultraviolet irradiation B; RA = Retinoic acid; HDF = normal human primary dermal fibroblast-neonatal cells. a $p<0.05$ as compared with UVB exposed cells.

IV. Discussion

Various functional cosmetics or ingredients have been developed for anti-aging, whitening, wrinkle free, moisturizing and skin protective purposes in market[41-43]. Currently available ingredients for functional cosmetics, however, have a number of limitations, such as economical problems, too expensive and exact or detail pharmacological and side effects are not fully understand[31]. Due to these factors, various efforts continuously attempt to search for affordable and effective functional ingredients with fewer side effects, especially on natural antioxidants[30,41-43]. In the present study, we intended to observe the anti-wrinkle (elastase and matrix metalloproteinase (MMP)-1 inhibition, and procollagen contents) and moisturizing (hyaluronan production) effects of PCP in *in vitro* test using HDF and HaCaT cells with possible cytotoxicity on HDF and HaCaT cells, as a process for developing the potent alternative anti-skin aging agent.

As results, no PCP treatment related significant cytotoxic effects were demonstrated against to the both HDF and HaCaT cells, from 0.75 to 4 mg/ml concentrations, the lowest to the highest concentrations tested in this experiment. PCP showed favorable free radical scavenging activities in dose-dependent manner. In PCP-treated HaCaT cells, hyaluronan synthesis was non-significantly but markedly increased, and pro-collagen productions were significantly increased in HDF cells, at all three different concentrations (0.25, 0.75 and 1 mg/ml), and elastase inhibitory activities were observed by PCP treatment. A significant decrease in UVB-induced MMP-1 activity was also observed in 1 mg/ml PCP-treated HDF cells as compared to those of UVB-exposed cells. Standard references - Vit C and E, TGF-β1, PPR, NAG, RA also showed favorable antioxidant, anti-wrinkle or moisturizing effects, ranged in reference values, respectively. Several studies demonstrated that Vitamin C and Vitamin E have strong free radical scavenging effect[41,51]. TGF-β, which appears in high concentrations in fibrotic cardiac tissue, is a potent inductor of tissue collagen deposition and of the differentiation of fibroblasts to myofibroblasts[52]. These properties of TGF-β make it possible to use as a

reference drug. PPR, one of elastase and metalloprotease inhibitor, was used as a reference drug[53]. Vit C is used due to strong elastase inhibitory effects[54]. NAG, one type of glucosamine, is the precursor to hyaluronic acid. NAG enhances the rate that skin fibroblasts make hyaluronic acid and collagen and reduce inflammation in skin[55,56]. Considering of these evidence, NAG is sufficient as a reference drug. Effect of RA is on reduction of collagen-degrading MMPs and RA has an inhibitory effect on metalloproteinases and delays the photoaging[57,58]. These results are considered as reliable evidences that PCP has favorable antioxidant, anti-wrinkle and moisturizing effects without meaningful cytotoxicity on HDF and HaCaT cell lines, at appropriate concentrations as serve as a predictable functional ingredient in the cosmetics, at least in a condition of the present *in vitro* assays.

Many studies have shown that pomegranates are rich in ellagic acid and polyphenols, which include flavonoids and hydrolyzable tannins[59-61]. Polyphenolics, contained in several plants, form the important part of the diet and exerts effective free radical scavenging and antioxidant activities. Studies have demonstrated that pomegranates exert strong anti-oxidant effects due to their free radical scavenging capacity and antioxidant properties[62,63]. These potent antioxidant properties may offer protection against skin aging. It is assumed that the combination of several polyphenol forms of pomegranate may exert various functions against skin aging. PCP used in this study contains ellagic acid (1.15 mg/g), and showed potent free radical scavenging activity in DPPH assays as comparable to those of positive controls, Vit C and Vit E, at the concentration of over 1.5 mg/ml, in the current experiment. Ellagic acid is a natural phenolic compound derived from many naturel sources and has been reported to show anti-carcinogenic[64], anti-fibrotic[65] and anti-oxidative effects[66]. It has been suggested that ellagic acid has a high affinity for copper at the active site of tyrosinase and inhibits its activity[67]. In addition, ellagic acid inhibited UV-induced skin pigmentation of guinea pigs[67]. Yoshimura et al.[61] has reported that a pomegranate containing 90% ellagic acid has skin-whitening effect *via* inhibition of the proliferation of melanocyte and melanin synthesis. We already reported that PCP effectively inhibited melanogenesis in B16F10 melanoma cells through p38 and PKA signaling pathways[43]. In addition, PCP also showed favorable *in vivo* skin protective activities against UVB-induced photoaging[44], and *in vivo* skin moisturizing effects through collagen and hyaluronan synthesis[45,46].

To evaluate the effects of PCP at cell based assays, we selected concentrations that did not affect cell viability as 1 mg/ml of highest levels. HDF cells and HaCaT cells were used for *in vitro* cell based experiments, since the skin comprises essentially three types of cells: Keratinocytes, melanocytes, and fibroblasts. Since hyaluronan, a major ECM component of the skin with collagen, plays important roles in skin physiology including water homeostasis[41,68,69], we next examined the effect the PCP on hyaluronan synthesis in HaCaT cells. As a result, PCP non-significantly, but remarkably increased hyaluronan synthesis in HaCaT cells. Skin aging is closely associated with a loss of skin moisture. It, therefore, expected that PCP will be showed favorable skin moisturizing effects through increase or enhance the hyaluronan synthesis, at least partially in a condition of this HaCaT cell base analysis, enough to serve as a predictable functional skin moisturizing cosmetic ingredient and inner beauty functional health food.

Type I collagen is the major structural protein in the skin. Collagen destruction is thought to underlie the appearance of aged skin and changes resulting from chronic sun exposure[70]. Skin wrinkle is a complex evitable process of human

life involves age-dependent decline of skin cell function. It causes harmful proteolytic degradation of ECM that leaves visible signs on the surface of the skin[37]. Several scientific evidences on skin wrinkle highlight the degradation of ECM, which is significantly associated with increased dermal enzymatic activities like hyaluronidase, collagenase, elastase and MMP-1 and formation of wrinkle[37,50]. 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 anti-wrinkle agent[1,71]. In addition, UVB radiation exposure to the skin results in the production of ROS implicated in MMP-1 activity responsible for collagen damage and photo-aging. Indirect evidence suggests that O_2 and H_2O_2 are major ROS related to UVB-mediated activation of MMP-1, MMP-2 and MMP-3[71-73]. MMP-mediated ECM damage has been shown to be a major contributor of photo-aging in human skin[41-43]. In the current analysis, elastase inhibitory activities were observed based on enzyme assay, and procollagen productions were examined in HDF cells. Moreover, characterized by alterations in the quantity of ECM, MMP-1 activity was evaluated in UVB-exposed HDF cells. Our data showed that UVB-induced MMP-1 activity was abrogated by appropriate concentrations of PCP, and pro-collagen synthesis were increased in HDF cells without significant cytotoxicity on HDF cells, upto reasonable concentrations of PCP. In addition, PCP effectively inhibited elastase activities at 1-2 mg/ml concentrations. It, therefore, was expected that PCP will be showed favorable anti-wrinkle effects on skin tissues without significant cytotoxic effects, at least in a condition of the present *in vitro* assays, enough to serve as a predictable functional anti-wrinkle cosmetic ingredient and inner beauty functional health food.

V. Conclusion

Taken together, the results of the present study suggest that PCP has favorable antioxidant, anti-wrinkle and moisturizing effects without meaningful cytotoxicity on HDF and HaCaT cell lines, at appropriate concentrations as serve as a predictable functional ingredient in the cosmetics and inner beauty functional health food, at least in a condition of the present in vitro assays. No PCP treatment related significant cytotoxic effects were demonstrated against to the both HDF and HaCaT cells, from 0.75 to 4 mg/ml concentrations, the lowest to the highest concentrations tested in this experiment. PCP showed favorable free radical scavenging activities in dose-dependent manner. In PCP-treated HaCaT cells, hyaluronan synthesis was non-significantly but markedly increased, and pro-collagen productions were significantly increased in HDF cells, at all three different concentrations (0.25, 0.75 and 1 mg/ml), and elastase inhibitory activities were observed by PCP treatment. A significant decrease in UVB-induced MMP-1 activity was also observed in 1 mg/ml PCP-treated HDF cells as compared to those of UVB-exposed cells. Standard references - Vit C and E, TGF-β1, PPR, RA also showed favorable antioxidant, anti-wrinkle or moisturizing effects, ranged in reference values, respectively.

Reference

1. Gilchrest BA. Skin aging and photoaging. Dermatology nursing. 1990;2(2):79-82.
2. Cevenini E, Invidia L, Lescai F, Salvioli S, Tieri P, Castellani G, Franceschi C. Human models of aging and longevity. Expert opinion on biological therapy. 2008;8(9):1393-1405.
3. Jurkiewicz BA, Buettner GR. Ultraviolet light-induced free radical formation in skin:

an electron paramagnetic resonance study. Photochemistry and photobiology. 1994;59(1): 1-4.
4. Messarah M, Boumendjel A, Chouabia A, Klibet F, Abdennour C, Boulakoud MS, Feki AE. Influence of thyroid dysfunction on liver lipid peroxidation and antioxidant status in experimental rats. Experimental and toxicologic pathology : official journal of the Gesellschaft fur Toxikologische Pathologie. 2010;62(3):301-310.
5. Rodriguez KJ, Wong HK, Oddos T, Southall M, Frei B, Kaur S. A purified feverfew extract protects from oxidative damage by inducing DNA repair in skin cells via a PI3-kinase-dependent Nrf2/ARE pathway. Journal of dermatological science. 2013;72(3):304-310.
6. Campanini MZ, Pinho-Ribeiro FA, Ivan AL, Ferreira VS, Vilela FM, Vicentini FT, Martinez RM, Zarpelon AC, Fonseca MJ, Faria TJ, Baracat MM, Verri WA, Jr., Georgetti SR, Casagrande R. Efficacy of topical formulations containing *Pimenta pseudocaryophyllus* extract against UVB-induced oxidative stress and inflammation in hairless mice. Journal of photochemistry and photobiology B, Biology. 2013;127:153-160.
7. Kim SR, Jung YR, An HJ, Kim DH, Jang EJ, Choi YJ, Moon KM, Park MH, Park CH, Chung KW, Bae HR, Choi YW, Kim ND, Chung HY. Anti-wrinkle and anti-inflammatory effects of active garlic components and the inhibition of MMPs via NF-kB signaling. PloS one. 2013;8(9):e73877.
8. Oh J, Kim JH, Park JG, Yi YS, Park KW, Rho HS, Lee MS, Yoo JW, Kang SH, Hong YD, Shin SS, Cho JY. Radical scavenging activity-based and AP-1-targeted anti-inflammatory effects of lutein in macrophage-like and skin keratinocytic cells. Mediators of inflammation. 2013;2013:787042.
9. Poswig A, Wenk J, Brenneisen P, Wlaschek M, Hommel C, Quel G, Faisst K, Dissemond J, Briviba K, Krieg T, Scharffetter-Kochanek K. Adaptive antioxidant response of manganese-superoxide dismutase following repetitive UVA irradiation. The Journal of investigative dermatology. 1999;112(1):13-18.
10. Fuchs J, Kern H. Modulation of UV-light-induced skin inflammation by D-alpha-tocopherol and L-ascorbic acid: a clinical study using solar simulated radiation. Free radical biology & medicine. 1998;25(9):1006-1012.
11. Kim YH, Chung CB, Kim JG, Ko KI, Park SH, Kim JH, Eom SY, Kim YS, Hwang YI, Kim KH. Anti-wrinkle activity of ziyuglycoside I isolated from a *Sanguisorba officinalis* root extract and its application as a cosmeceutical ingredient. Bioscience, biotechnology, and biochemistry. 2008;72(2):303-311.
12. Hunt G, Kyne S, Ito S, Wakamatsu K, Todd C, Thody A. Eumelanin and phaeomelanin contents of human epidermis and cultured melanocytes. Pigment cell research. 1995;8(4): 202-208.
13. Vincensi MR, d'Ischia M, Napolitano A, Procaccini EM, Riccio G, Monfrecola G, Santoianni P, Prota G. Phaeomelanin versus eumelanin as a chemical indicator of ultraviolet sensitivity in fair-skinned subjects at high risk for melanoma: a pilot study. Melanoma research. 1998;8(1):53-58.
14. Kvam E, Dahle J. Pigmented melanocytes are protected against ultraviolet-A-induced membrane damage. The Journal of investigative dermatology. 2003;121(3):564-569.
15. Yamazaki F, Okamoto H, Miyauchi-Hashimoto H, Matsumura Y, Itoh T, Tanaka K, Kunisada T, Horio T. XPA gene-deficient, SCF-transgenic mice with epidermal melanin are resistant to UV-induced carcinogenesis. The Journal of investigative dermatology. 2004;123(1):220-228.

16. Wagner JK, Parra EJ, H LN, Jovel C, Shriver MD. Skin responses to ultraviolet radiation: effects of constitutive pigmentation, sex, and ancestry. Pigment cell research. 2002;15(5): 385-390.
17. Ortonne JP. Photoprotective properties of skin melanin. The British journal of dermatology. 2002;146 Suppl 61:7-10.
18. Yamaguchi Y, Takahashi K, Zmudzka BZ, Kornhauser A, Miller SA, Tadokoro T, Berens W, Beer JZ, Hearing VJ. Human skin responses to UV radiation: pigment in the upper epidermis protects against DNA damage in the lower epidermis and facilitates apoptosis. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 2006;20(9):1486-1488.
19. Tada M, Kohno M, Niwano Y. Scavenging or quenching effect of melanin on superoxide anion and singlet oxygen. Journal of clinical biochemistry and nutrition. 2010;46(3):224-228.
20. Ito S, Wakamatsu K. Chemistry of mixed melanogenesis--pivotal roles of dopaquinone. Photochemistry and photobiology. 2008;84(3): 582-592.
21. Khazaeli P, Goldoozian R, Sharififar F. An evaluation of extracts of five traditional medicinal plants from Iran on the inhibition of mushroom tyrosinase activity and scavenging of free radicals. International journal of cosmetic science. 2009;31(5):375-381.
22. Momtaz S, Mapunya BM, Houghton PJ, Edgerly C, Hussein A, Naidoo S, Lall N. Tyrosinase inhibition by extracts and constituents of *Sideroxylon inerme* L. stem bark, used in South Africa for skin lightening. Journal of ethnopharmacology. 2008;119(3): 507-512.
23. Ng LT, Ko HH, Lu TM. Potential antioxidants and tyrosinase inhibitors from synthetic polyphenolic deoxybenzoins. Bioorganic & medicinal chemistry. 2009;17(13):4360-4366.
24. Nugroho A, Choi JK, Park JH, Lee KT, Cha BC, Park HJ. Two new flavonol glycosides from *Lamium amplexicaule* L. and their *in vitro* free radical scavenging and tyrosinase inhibitory activities. Planta medica. 2009; 75(4):364-366.
25. Wang KH, Lin RD, Hsu FL, Huang YH, Chang HC, Huang CY, Lee MH. Cosmetic applications of selected traditional Chinese herbal medicines. Journal of ethnopharmacology. 2006;106(3):353-359.
26. Solomon EI, Chen P, Metz M, Lee SK, Palmer AE. Oxygen Binding, Activation, and Reduction to Water by Copper Proteins. Angewandte Chemie (International ed in English). 2001; 40(24):4570-4590.
27. Chen P, Solomon EI. O_2 activation by binuclear Cu sites: noncoupled versus exchange coupled reaction mechanisms. Proceedings of the National Academy of Sciences of the United States of America. 2004;101(36):13105-13110.
28. Inoue T, Shiota Y, Yoshizawa K. Quantum chemical approach to the mechanism for the biological conversion of tyrosine to dopaquinone. Journal of the American Chemical Society. 2008;130(50):16890-16897.
29. Rob D. Tyrosinase. In: Lontie R, editor. Copper Proteins and Copper Enzymes. IIth ed. Florida: CRC Press; 1984.p.207-240.
30. Cho EA, Cho EH, Choi SJ, Park KH, Kim SY, Jeong YJ, Ku CS, Ha BJ, Jang DI, Chae HJ. Screening of anti-wrinkle resource from herbal medicinal extracts and stability test of its cosmetic products. Korean J Medicinal Crop Sci. 2011;19(2):126-135.
31. Kwak YJ, Lee DH, Kim NM, Lee JS. Screening and extraction condition of anti-skin aging elastase inhibitor from medicinal plants. Korean J Medicinal Crop Sci. 2005;13(6):213-216.
32. Wiedow O, Schröder JM, Gregory H, Young

JA, Christophers E. Elafin: an elastase-specific inhibitor of human skin. Purification, characterization, and complete amino acid sequence. The Journal of biological chemistry. 1990; 265(25):14791-14795.

33. Gil MI, Tomás-Barberán FA, Hess-Pierce B, Holcroft DM, Kader AA. Antioxidant activity of pomegranate juice and its relationship with phenolic composition and processing. Journal of agricultural and food chemistry. 2000;48(10):4581-4589.
34. Noda Y, Kaneyuki T, Mori A, Packer L. Antioxidant activities of pomegranate fruit extract and its anthocyanidins: delphinidin, cyanidin, and pelargonidin. Journal of agricultural and food chemistry. 2002;50(1):166-171.
35. Singh RP, Chidambara Murthy KN, Jayaprakasha GK. Studies on the antioxidant activity of pomegranate (*Punica granatum*) peel and seed extracts using in vitro models. Journal of agricultural and food chemistry. 2002; 50(1):81-86.
36. Kim ND, Mehta R, Yu W, Neeman I, Livney T, Amichay A, Poirier D, Nicholls P, Kirby A, Jiang W, Mansel R, Ramachandran C, Rabi T, Kaplan B, Lansky E. Chemopreventive and adjuvant therapeutic potential of pomegranate (*Punica granatum*) for human breast cancer. Breast cancer research and treatment. 2002; 71(3):203-217.
37. Maity N, Nema NK, Abedy MK, Sarkar BK, Mukherjee PK. Exploring Tagetes erecta Linn flower for the elastase, hyaluronidase and MMP-1 inhibitory activity. Journal of ethnopharmacology. 2011;137(3):1300-1305.
38. Chan YY, Kim KH, Cheah SH. Inhibitory effects of *Sargassum polycystum* on tyrosinase activity and melanin formation in B16F10 murine melanoma cells. Journal of ethnopharmacology. 2011;137(3):1183-1188.
39. Imokawa G. Recent advances in characterizing biological mechanisms underlying UV-induced wrinkles: a pivotal role of fibrobrast-derived elastase. Arch dermatol Res. 2008;300(1):7-20.
40. Kim EJ, Kim MK, Jin XJ, Oh JH, Kim JE, Chung JH. Skin aging and photoaging alter fatty acids composition, including 11,14,17-eicosatrienoic acid, in the epidermis of human skin. Journal of Korean medical science. 2010;25(6):980-983.
41. Kang SJ, Choi BR, Kim SH, Yi HY, Park HR, Park SJ, Song CH, Park JH, Lee YJ, Kwang S. Inhibitory effects of pomegranate concentrated solution on the activities of hyaluronidase, tyrosinase, and metalloproteinase. Journal of cosmetic science. 2015; 66(3):145-159.
42. Kang SJ, Choi BR, Kim SH, Yi HY, Park HR, Song CH, Park SJ, Ku SK, Lee YJ. Effect of pomegranate concentration solution on photoaging. J Soc Prev Korean Med. 2015; 19:109-116.
43. Kang SJ, Choi BR, Lee EK, Kim SH, Yi HY, Park HR, Song CH, Lee YJ, Ku SK. Inhibitory effect of dried pomegranate concentration powder on melanogenesis in B16F10 melanoma cells; involvement of p38 and PKA signaling pathways. International journal of molecular sciences. 2015;16(10):24219-24242.
44. Kang SJ, Choi BR, Kim SH, Yi HY, Park HR, Song CH, Ku SK, Lee YJ. Beneficial effects of dried pomegranate juice concentrated powder on ultraviolet B-induced skin photoaging in hairless mice. Experimental and therapeutic medicine. 2017;14(2):1023-1036.
45. Hu JR, Choi BR, Park HR, Sung MS, Yi HY, Kang SJ, Ku SK, Lee YJ. Skin tissue homogenate analysis for ceramide and TGF-1 contents with TGF-1 mRNA expressions after treatment of pomegranate concentrated solution and dried pomegranate concentrate

powder in mice. J Korean Med. 2016;37(4):1-9.
46. Kang SJ, Choi BR, Kim SH, Yi HY, Park HR, Sung MS, Song CH, Cho IJ, Lee YJ, Ku SK. Evaluation of the *in vivo* skin moisturizing effects and underlying mechanisms of pomegranate concentrate solution and dried pomegranate concentrate powder. J Korean Med. 2016;37(2):12-22.
47. Blois MS. Antioxidant determinations by the use of a stable free radical. Nature. 1958;181(4617):1199.
48. Jiang P, Li X, Thompson CB, Huang Z, Araiza F, Osgood R, Wei G, Feldmann M, Frost GI, Shepard HM. Effective targeting of the tumor microenvironment for cancer therapy. Anticancer research. 2012;32(4):1203-1212.
49. Heo JC, Park JY, An SM, Lee JM, Yun CY, Shin HM, Kwon TK, Lee SH. Anti-oxidant and anti-tumor activities of crude extracts by *Gastrodia elata Blume*. Korean J Food Preserv. 2006;13(1):83-87.
50. Losso JN, Munene CN, Bansode RR, Bawadi HA. Inhibition of matrix metalloproteinase-1 activity by the soybean Bowman-Birk inhibitor. Biotechnology letters. 2004;26(11):901-905.
51. Chen AY, Lu JM, Yao Q, Chen C. Entacapone is an Antioxidant More Potent than Vitamin C and Vitamin E for Scavenging of Hypochlorous Acid and Peroxynitrite, and the Inhibition of Oxidative Stress-Induced Cell Death. Medical science monitor : international medical journal of experimental and clinical research. 2016;22:687-696.
52. Lijnen P, Petrov V. Transforming growth factor-beta 1-induced collagen production in cultures of cardiac fibroblasts is the result of the appearance of myofibroblasts. Methods and findings in experimental and clinical pharmacology. 2002;24(6):333-344.
53. Morihara K, Tsuzuki H. Phosphoramidon as an inhibitor of elastase from *Pseudomonas aeruginosa*. The Japanese journal of experimental medicine. 1978;48(1):81-84.
54. Kim DW, Kim HJ, Chae HS, Park NG, Kim YB, Jang A. Anti-oxidation and Anti-wrinkling Effects of Jeju Horse Leg Bone Hydrolysates. Korean journal for food science of animal resources. 2014;34(6):844-851.
55. Kikuchi K, Matahira Y. Oral N-acetylglucosamine supplementation improves skin conditions of female volunteers: Clinical evaluation by a microscopic three-dimensional skin surface analyzer. Journal of applied cosmetology. 2002;20(2):143-152.
56. Uitterlinden EJ, Koevoet JL, Verkoelen CF, Bierma-Zeinstra SM, Jahr H, Weinans H, Verhaar JA, van Osch GJ. Glucosamine increases hyaluronic acid production in human osteoarthritic synovium explants. BMC musculoskeletal disorders. 2008;9:120.
57. Lateef H, Stevens MJ, Varani J. All-trans-retinoic acid suppresses matrix metalloproteinase activity and increases collagen synthesis in diabetic human skin in organ culture. The American journal of pathology. 2004;165(1):167-174.
58. Jurzak M, Latocha M, Gojniczek K, Kapral M, Garncarczyk A, Pierzchala E. Influence of retinoids on skin fibroblasts metabolism *in vitro*. Acta poloniae pharmaceutica. 2008;65(1):85-91.
59. Afaq F, Saleem M, Krueger CG, Reed JD, Mukhtar H. Anthocyanin- and hydrolyzable tannin-rich pomegranate fruit extract modulates MAPK and NF-kB pathways and inhibits skin tumorigenesis in CD-1 mice. International journal of cancer. 2005;113(3):423-433.
60. Wang RF, Xie WD, Zhang Z, Xing DM, Ding Y, Wang W, Ma C, Du LJ. Bioactive compounds from the seeds of *Punica granatum* (pomegranate). Journal of natural products. 2004;67(12):2096-2098.
61. Yoshimura M, Watanabe Y, Kasai K, Yamakoshi

J, Koga T. Inhibitory effect of an ellagic acid-rich pomegranate extract on tyrosinase activity and ultraviolet-induced pigmentation. Bioscience, biotechnology, and biochemistry. 2005;69(12):2368-2373.
62. Ross JA, Kasum CM. Dietary flavonoids: bioavailability, metabolic effects, and safety. Annual review of nutrition. 2002;22:19-34.
63. Soobrattee MA, Bahorun T, Aruoma OI. Chemopreventive actions of polyphenolic compounds in cancer. BioFactors (Oxford, England). 2006;27(1-4):19-35.
64. Maas JL, Galletta GJ, Stoner GD. Ellagic acid, an anticarcinogen in fruits, especially in strawberries: a review. HortScience. 1991; 26(1):10-14.
65. Thresiamma KC, Kuttan R. Inhibition of liver fibrosis by ellagic acid. Indian journal of physiology and pharmacology. 1996;40(4): 363-366.
66. Osawa T, Ide A, Su JD, Namiki M. Inhibition of in vitro lipid peroxidation by ellagic acid. Journal of agricultural and food chemistry. 1987;35(5):808-812.
67. Shimogaki H, Tanaka Y, Tamai H, Masuda M. *In vitro* and in vivo evaluation of ellagic acid on melanogenesis inhibition. International journal of cosmetic science. 2000;22(4):291-303.
68. Yamane T, Nakagami G, Yoshino S, Muramatsu A, Matsui S, Oishi Y, Kanazawa T, Minematsu T, Sanada H. Hydrocellular foam dressing promotes wound healing along with increases in hyaluronan synthase 3 and PPARa gene expression in epidermis. PloS one. 2013; 8(8):e73988.
69. Frenkel JS. The role of hyaluronan in wound healing. International wound journal. 2014; 11(2):159-163.
70. Liao PL, Li CH, Chang CY, Lu SR, Lin CH, Tse LS, Cheng YW. Anti-ageing effects of alpha-naphthoflavone on normal and UVB-irradiated human skin fibroblasts. Experimental dermatology. 2012;21(7):546-548.
71. Kim HD, Cho HR, Moon SB, Shin HD, Yang KJ, Park BR, Jang HJ, Kim LS, Lee HS, Ku SK. Effects of beta-glucan from *Aureobasidium pullulans* on acute inflammation in mice. Archives of pharmacal research. 2007;30(3): 323-328.
72. Adams LS, Seeram NP, Aggarwal BB, Takada Y, Sand D, Heber D. Pomegranate juice, total pomegranate ellagitannins, and punicalagin suppress inflammatory cell signaling in colon cancer cells. Journal of agricultural and food chemistry. 2006;54(3):980-985.
73. Orgil O, Schwartz E, Baruch L, Matityahu I, Mahajna J, Amir R. The antioxidative and anti-proliferative potential of non-edible organs of the pomegranate fruit and tree. LWT-Food Science and Technology. 2014; 58(2):571-577.