

## *In vitro* Effects of the Complex of Korean *Citrus junos* and Medicinal Herbs on the Cell Growth in CCD-986sk Human Fibroblast Cell Line

Kap Joo Park\*

Department of Biological Sciences, Kon-Kuk University, Seoul 143-701, Korea

**Abstract** - In order to investigate whether or not CCD-986sk cell line can be affected by Korean *Citrus junos* and medicinal herbs, we examined the MTT assay when we treated Korean *Citrus junos* and medicinal herbs in CCD-986sk human fibroblast cell line. The samples that added complex of grinded extracts of Korean *Citrus junos* and boiling-water extracts from Korean medicinal herbs were tested for cell proliferation activity by means of a modification of the MTT assay. Among mixture of Citron 3 (Citron 3, less mellowed citron which was ripened for three months) and boiling-water extracts, the group Citron 3+*Phellinus linteus* showed significantly strong cell proliferation activity. And among mixture of Citron 4 (Citron 4, completely mellowed citron which was ripened for four months) and boiling-water extracts, the group Citron 4+*Cordyceps militaris* and Citron 4+*Phellinus linteus* showed significantly strong cell proliferation activity, respectively. These results suggest that complex of Korean *Citrus junos* and medicinal herbs could be an excellent candidate for protection of human skin aging.

**Key words** : Korea *Citrus junos*, medicinal herbs, CCD-986sk human fibroblast cell line, MTT assay

### INTRODUCTION

With advancing age, the skin and its appendages undergo marked changes. Alterations include increased roughness, wrinkling, loss of elasticity, mottling, and a general transparency or thinness (Lavker *et al.* 1987). The skin aging divide into the intrinsic and the extrinsic aging. The intrinsic aging is a natural aging that is the decline of the skin structure and the physiological function continuously. The extrinsic aging is caused by ultraviolet exposure or stress. The ultraviolet is well known one of aging factors. It has been studied the photoaging mechanism *in vivo*. As the skin is exposed to ultraviolet rays, the collagen and elastin are denatured.

And then horny layer is thickened and wrinkled. In the studies about the intrinsic aging, Han's research (1998) showed histological changes in the skin with innate aging. Choi's research (1997) reported on evaluation of skin furrows in the aging process using an image analysis system. In contrast to Han and Choi, Koo (1998) and his colleague studied the extrinsic aging in skin, they reported on the effect ultraviolet radiation in aging. And Lavker (1987) and Braveman (1982) reported on the study about aged skin by light, transmission electron, and scanning electron microscopy. Lavker (1987) showed the difference between cell state of young and old persons. Braveman (1982) showed the difference between sun exposed and sun protected skin.

The ultraviolet radiation in the above-mentioned extrinsic aging increase a pigmentary deposit. Such an irradiation induce the melanin cell to increase in num-

\* Corresponding author: Kap Joo Park, Tel. 02-447-5018, Fax. 02-3436-5432, E-mail. kkupkj@konkuk.ac.kr

ber. Kim (1988) showed the effect of UV-B radiation on epidermal melanocytes of C57BL mouse. And Han (1987) reported on the effect of sulfhydryl compounds, cysteine and glutathione on the size of melanosomes and the ratio of melanosomal stages of epidermal melanocytes in UV-irradiated black mice.

As have been seen above, scientists have studied the skin aging and discovered materials that prevent the skin aging. It is reported that these materials is the acceleration of collagens, the proliferation of fibroblasts, and the expansion of ECMs (extracellular matrix). Lee *et al.* (1997) reported that L-Ascorbic acid and its inducer have an effect on antioxidant, accelerating fibroblast growth, and increasing collagen biosynthesis. Kang *et al.* (1996) reported on action of retinoids and vitamin D in skin. Kim *et al.* (1989) studied effects of Panax Ginseng and Radix Astragali on age-related physiological alterations in rats.

As the mentioned researches, there have been numerous attempts to develop clinically useful compounds to protect or cure skin aging. However, it is well documented that a lot of chemical compounds may exhibit severe cytotoxicity, reproductive toxicity and other important side effects. The chemical compounds are taken through the mouth and skin. Some of them are carried to the liver through the front hepatic portal vein, most of them are regarded as extraneous substances, and are removed within the liver, and some of them are excreted through the kidney. So most of taken chemical materials effects the liver (Chait *et al.* 1972). Therefore, in order to find an alternative to the traditional cure, studies have increasingly focused on the development of therapeutic agents based on natural products and medicinal herbs.

In this study, we investigated whether the complex of Korean *Citrus junos* and medicinal herbs protects skin aging at the *in vitro* level, thereby resulting in protection from increased roughness, wrinkling, loss of elasticity, mottling, and a general transparency or thinness (Lavker *et al.* 1987). The each complex of Korean *Citrus junos* and medicinal herbs were treated to the CCD-986sk cell line monolayer (human fibroblast, KCBL-21947). The protective effect of each complex of Korean *Citrus junos* and medicinal herbs was examined by means of a modification of the MTT (3-[4, 5-dimethyl-

thiazol-2-yl]-2, 5-diphenyl tetrazolium bromide) assay (Kang and Park 1997). The major finding of this paper is that Citron 3+*Phellinus linteus*, Citron 4+*Cordyceps militaris* and Citron 4+*Phellinus linteus* are skin-aging protective and ameliorates intrinsic or extrinsic-mediated skin-aging at *in vitro* level.

## MATERIALS AND METHODS

### 1. Cell and media

The CCD-986sk cell line (human skin fibroblast, KCBL-21947) was obtained from Korean Cell Line Bank (KCLB; Seoul, Korea). CCD-986sk cell line was grown as a monolayer culture in 75 cm<sup>2</sup> plastic tissue culture flasks (Nunc, USA) in Dulbecco's modified Eagle's medium (DMEM; GIBCO, USA) with 10% fetal bovine serum (FBS; Hyclone, USA), 0.22% sodium bicarbonate (Sigma, USA). This cell line was maintained at 37°C in a 5% CO<sub>2</sub> incubator.

### 2. Preparation of Korean *Citrus junos* and medicinal herb extracts

Korean *Citrus junos* and medicinal herbs were prepared on the basis of a review of Korean traditional medicine books (Yoon 1992; Huh 1981) and on the basis of recommendation of Korean traditional medical doctors. Common and scientific names of herbs are shown in Table 1.

All *Citrus junos* used were purchased from Seungil farm (Wando, Korea) at October (ripened for three months) of 2002 and November (ripened for four months) of 2002. Less mellowed citron (ripened for three months, so not mellowed completely, afterward it will be marked C3) and mellowed citron (ripened for four months, so mellowed completely, it'll be marked by C4) were grinded properly by using mixer (Hanil Co. Ltd., Korea). The grinded samples were centrifuged at 6,000 × g for 15 minutes and supernatant juice were filtered by using gauze. And the samples were vacuum concentrated by using rotavapo R-200 (Buchi, Germany), and lyophilized. The each 1 g of dried sample was dissolved in the 5 ml of 0.8% methanol (concentrated sample) and filtered through membranes of 0.45 μm pore size (Millipore,

**Table 1.** List of Korean medicinal herbs

| No. | Family and species                                       | Voucher no. | Vernacular name (s) | Efficacy after treatment  |
|-----|--|-------------|---------------------|---|
| 1   | Labiatae<br><i>Agastache rugosa</i>                      | 1-1k        | KwakYang            | Aerial part used to dispel summer heat and dampness   |
| 2   | Leguminosae<br><i>Phaseolus radiatus</i> L.              | 1-2k        | NokDu               | Fruits used as cutaneous disease  |
| 3   | Umbelliferae<br><i>Angelica gigas</i> Nakai              | 1-3k        | TangGwi             | Root juice used to nourish the blood and to invigorate the blood circulation                          |
| 4   | Cordycepitateae<br><i>Cordyceps militaris</i>            | 1-4k        | DongChungHaCho      | Fruit body used as an antidote and anticancer drug  |
| 5   | Labiatae<br><i>Mentha haplocalyx</i> Briq.               | 1-5k        | PaKa                | Aerial part used for affection due to wind and heat with fever, headache, stuffy nose and sore throat |
| 6   | Araceae<br><i>Pinellia ternata</i>                       | 1-6k        | PanHa               | Tuberous roots used for the treatment of emesis and diarrhea  |
| 7   | Hymenochaetaceae<br><i>Phellinus linteus</i>             | 1-7k        | SangHwang           | Fruit body used as an anticancer, anti-skin aging drug and antidote                                   |
| 8   | Scrophulariaceae<br><i>Rehmannia glutinosa</i>           | 1-8k        | SaengGiHwang        | Tuberous roots used for the treatment of thirst, exanthesis and bleeding                              |
| 9   | Ranunculaceae<br><i>Cimicifuga heracleifolia</i> Kom.    | 1-9k        | SeungMa             | Root juice used as an antipyretic and antidote  |
| 10  | Betulaceae<br><i>Alnus japonica</i>                      | 1-10k       | OhRi                | Bark used as an antipyretic and styptic   |
| 11  | Ranunculaceae<br><i>Paeonia lactiflora</i> Pall.         | 1-11k       | ChagYak             | Root juice used to nourish the blood and pain-relieving agent for headache                            |
| 12  | Compositae<br><i>Xanthium strumarium</i> L.              | 1-12k       | ChangYiJa           | Fruits used to treat cough and fever  |
| 13  | Compositae<br><i>Atractylodes japonica</i> Koidz.        | 1-13k       | ChangChul           | Rhizome juice used for treating dyspepsia due to accumulation of dampness                             |
| 14  | Rubiaceae<br><i>Gardenia jasminoides</i> Ellis.          | 1-14k       | ChiCha              | Ripe fruit juice used as antipyretic and sedative for fever with fidgetiness                          |
| 15  | Polygonaceae<br><i>Polygonum multiflorum</i> Thunb.      | 1-15k       | HaSuOh              | Tuberous roots used for robustness  |
| 16  | Rosaceae<br><i>Prunus armeniaca</i> L.                   | 1-16k       | HaengIn             | Seeds used to relieve cough   |
| 17  | Cyperaceae<br><i>Cyperus rotundus</i> L.                 | 1-17k       | HyangPuJa           | Tuberous roots used to treat headache and stomachache   |
| 18  | Scrophulariaceae<br><i>Scrophularia buergeriana</i> Miq. | 1-18k       | HyonSam             | Root juice used to treat sore throat and swelling   |
| 19  | Labiatae<br><i>Scutellaria baicalensis</i> Georgi        | 1-19k       | HwangGum            | Root juice used to eliminate heat in the lung for cough with yellow thick phlegm                      |
| 20  | Ranunculaceae<br><i>Coptis japonica</i> Makino           | 1-20k       | HwangRyon           | Rhizome juice used as antiphlogistic for various infections   |

France) and used samples.

Each medicinal herb extracts was prepared from dried Korean medicinal herbs. Each volume (100 g) of every herb was added to 1200 ml of sterilized water, and boiled for 150 min by using herbal medicine decocter (Daewoong Co. Ltd., Seoul). The aqueous extracts from each sample were centrifuged at 6000 × g for 15 min and filtered through 3MM filter papers (Whatman, England). And the samples were vacuum concentrated by using rotavapo R-200 (Buchi, Germany), and lyophilized. The 1 g of dried samples were dissolved in the 0.5 ml of 0.8%

methanol (concentrated sample) and filtered through membranes of 0.45 μm pore size (Millipore, France) and used samples. All Samples were stored at 4°C.

The following terminology was used for the extracts: the first letter C3 represents juice of *Citrus junos* which ripened for three months, so not mellowed completely and C4 represents juice of *Citrus junos* which ripened for four months, so mellowed completely, while the second arabic numerals represent boiling water extracts of each Korean herb as shown in Table 1. For example, C3-1 represents the complex of C3 juice plus boiling

water extract of *Agastache rugosa*.

### 3. Treatment of each Korean *Citrus junos* and medicinal herb extracts to CCD-986sk cell lines

Each complex of Korean *Citrus junos* and medicinal herb extracts in a ratio of one to one used in this experiment. These samples were added to 96-well culture plates containing  $1 \times 10^5$  CCD-986sk cells per well. Each extract (100  $\mu$ l, added citron and medicinal herb at the concentration of 200 mg ml<sup>-1</sup>, respectively) was diluted sequentially in two-fold steps with PBS and then added to each well. PBS and 0.8% methanol was diluted in two-fold steps and then added to each well (normal control). The culture plates were then incubated at 37°C, in a 5% CO<sub>2</sub> incubator for 2 days and used in estimation of cell proliferation effect, by means of the MTT (tertrazolium-based colorimetric) assay.

### 4. MTT (Tetrazolium-Based Colorimetric) Assay Method

A modification of the MTT assay was used, by means of the cell proliferation kit 1 (MTT, Roche, Germany). 96-Well culture plates containing cell and extracts from each specimen were cultured for 2 days. 10  $\mu$ l of MTT (5 mg ml<sup>-1</sup> in phosphate buffered saline) were added to each well. After a further 4 h incubation, 100  $\mu$ l of solubilization solution (10% SDS in 0.01 M HCl) was added to each well. The absorbance (test wavelength 560 nm, reference wavelength of >650 nm) of each well was measured using an ELISA plate reader (Molecular Devices, USA). Subsequently, data were stored and analyzed through the software program.

### 5. Statistical analysis

All results were shown as mean  $\pm$  standard deviation. Statistical evaluation of data was performed by Student t-test to make comparisons between groups.

## RESULT AND DISCUSSION

### 1. Proliferation effect of the complex of C3 and Korean medicinal herb extracts on the CCD-986sk human fibroblast cell lines

The complexes of C3 and Korean medicinal herb extracts were screened for their activated effect on CCD-986sk cells by means of MTT assay. Sequentially diluted PBS and 0.8% methanol was used normal control group. The same test was done four times and statistical evaluation of data was performed by student t-test.

The aim of this study was to investigate the anti skin-aging effects of Korean *citrus junos* and medicinal herbs at *in vitro* level. The effect of some complexes of Korean *citrus junos* and medicinal herbs on the CCD-986sk human fibroblast cell lines was clearly powerful as shown in Table 2. The C3-7 (Citron 3+*Phellinus linteus*) showed strong cell proliferation activity compared with the normal control group at the concentration of <50 mg ml<sup>-1</sup>. While C3-15 (Citron 3+*Polygonum multiflorum*) showed strong cell proliferation activity at the concentration of <25 mg ml<sup>-1</sup>. As have been seen above, C3-7 and C3-15 showed strong cell proliferation activity more than 105 percent to 244 percent compared with the normal control group, in the view of cell proliferation ratio. Based on reports (Lavker

**Table 2.** Effects of the complex of C3 and medicinal herb extracts on the CCD-986sk human fibroblast cell growth

| Groups   | Optical densities at 560 nm at the following concentration (mg ml <sup>-1</sup> ) of the extracts |                               |                              |                              |                                |                                |                                |                                |
|----------|---|-------------------------------|------------------------------|------------------------------|--------------------------------|--------------------------------|--------------------------------|--------------------------------|
|          | 200<br>(mg ml <sup>-1</sup> )   | 100<br>(mg ml <sup>-1</sup> ) | 50<br>(mg ml <sup>-1</sup> ) | 25<br>(mg ml <sup>-1</sup> ) | 12.5<br>(mg ml <sup>-1</sup> ) | 6.25<br>(mg ml <sup>-1</sup> ) | 3.13<br>(mg ml <sup>-1</sup> ) | 1.56<br>(mg ml <sup>-1</sup> ) |
| PBS      | 0.068 $\pm$ 0.002   | 0.068 $\pm$ 0.002             | 0.068 $\pm$ 0.002            | 0.068 $\pm$ 0.002            | 0.068 $\pm$ 0.002              | 0.068 $\pm$ 0.002              | 0.068 $\pm$ 0.002              | 0.068 $\pm$ 0.002              |
| Methanol | 0.068 $\pm$ 0.003   | 0.068 $\pm$ 0.003             | 0.068 $\pm$ 0.003            | 0.068 $\pm$ 0.003            | 0.068 $\pm$ 0.003              | 0.068 $\pm$ 0.003              | 0.068 $\pm$ 0.003              | 0.068 $\pm$ 0.003              |
| C3-7     | 0.057 $\pm$ 0.001   | 0.046 $\pm$ 0.002             | 0.146 $\pm$ 0.001*           | 0.112 $\pm$ 0.001*           | 0.088 $\pm$ 0.001*             | 0.079 $\pm$ 0.000*             | 0.072 $\pm$ 0.001*             | 0.065 $\pm$ 0.001              |
| C3-15    | 0.041 $\pm$ 0.001   | 0.061 $\pm$ 0.003             | 0.078 $\pm$ 0.007            | 0.121 $\pm$ 0.002*           | 0.129 $\pm$ 0.002*             | 0.088 $\pm$ 0.010*             | 0.100 $\pm$ 0.028              | 0.097 $\pm$ 0.005*             |

All values are mean  $\pm$  S.D. (standard deviation) of four times tests.

"\*" values are significantly different from the control at P<0.1 as determined by student t-test. C3-arabic numerals were composed of C3 plus Korean herb extracts in a ratio of one to one. C3 indicates extract of citron for ripened 3 months. Each arabic numeral indicates Korean herb extracts as shown in Table 1.

**Table 3.** Effects of 4 months ripened *Citrus junos* and Korean medicinal herb extracts on the CCD-986sk human fibroblast cell growth

| Groups   | Optical densities at 560 nm at the following concentration (mg ml <sup>-1</sup> ) of the extracts |                               |                              |                              |                                |                                |                                |                                |
|----------|---|-------------------------------|------------------------------|------------------------------|--------------------------------|--------------------------------|--------------------------------|--------------------------------|
|          | 200<br>(mg ml <sup>-1</sup> )   | 100<br>(mg ml <sup>-1</sup> ) | 50<br>(mg ml <sup>-1</sup> ) | 25<br>(mg ml <sup>-1</sup> ) | 12.5<br>(mg ml <sup>-1</sup> ) | 6.25<br>(mg ml <sup>-1</sup> ) | 3.13<br>(mg ml <sup>-1</sup> ) | 1.56<br>(mg ml <sup>-1</sup> ) |
| PBS      | 0.068±0.002   | 0.068±0.002                   | 0.068±0.002                  | 0.068±0.002                  | 0.068±0.002                    | 0.068±0.002                    | 0.068±0.002                    | 0.068±0.002                    |
| Methanol | 0.068±0.003   | 0.068±0.003                   | 0.068±0.003                  | 0.068±0.003                  | 0.068±0.003                    | 0.068±0.003                    | 0.068±0.003                    | 0.068±0.003                    |
| C4-7     | 0.054±0.000   | 0.044±0.002                   | 0.099±0.002*                 | 0.108±0.001*                 | 0.087±0.000*                   | 0.079±0.000*                   | 0.072±0.001*                   | 0.065±0.001                    |
| C4-8     | 0.039±0.001   | 0.052±0.001                   | 0.107±0.057                  | 0.094±0.024                  | 0.109±0.044                    | 0.111±0.019*                   | 0.128±0.013*                   | 0.120±0.018*                   |
| C4-15    | 0.037±0.000   | 0.058±0.007                   | 0.121±0.042                  | 0.101±0.010*                 | 0.135±0.037*                   | 0.132±0.026*                   | 0.121±0.037*                   | 0.148±0.043*                   |

All values are mean±S.D. (standard deviation) of four times tests.

"\*" values are significantly different from the control at  $P < 0.1$  as determined by student t-test. C4-arabic numerals were composed of C4 plus Korean herb extracts in a ratio of one to one. C4 indicates extract of citron for ripened 4 months. Each arabic numeral indicates Korean herb extracts as shown in Table 1.

*et al.* 1987; Han 1998; Choi 1997) indicating that an elevated intrinsic problems (decline of the skin structure and the physiological function) and extrinsic problems (ultraviolet exposure or stress) is one of the main causes of skin aging, a number of drugs have been developed to renature the skin collagen and elastin and to protect wrinkle of skin horny layer. However, little work has been done in developing natural materials to prevent skin-aging. In this context, this report suggests that some complexes of Korean *Citrus junos* and medicinal herbs may represent an alternative therapeutic agent to assist in the prevention and treatment of skin-aging.

## 2. Proliferation effect of the complex of C4 and Korean medicinal herb extracts on the CCD-986sk human fibroblast cell lines

The complexes of C4 and medicinal herb extracts in a ratio of one to one were screened for their effect on CCD-986sk cells by means of MTT assay. The same test was done four times and statistical evaluation of data was performed by student t-test.

As shown in Table 3, the CCD-986sk human fibroblast cell proliferation activity of C4-7 (Citron 4+*Phellinus linteus*), C4-8 (Citron 4+*Rehmannia glutinosa*) and C4-15 (Citron 4+*Polygonum multiflorum* Thunb.) showed strong cell proliferation activity compared with the normal control group at the concentration of  $< 50$  mg ml<sup>-1</sup>. As a results, the complex of C3 and C4 plus *Phellinus linteus* and the complex of C3 and C4 plus *Polygonum multiflorum* Thunb. exhibited strong CCD-986sk cell proliferation activity at *in vitro* level. These results suggest that the complex of Korean *Citrus junos*

and *Phellinus linteus*/*Polygonum multiflorum* Thunb. could be a promising candidate for protection of human skin aging.

According to Korean traditional medicine books, fruit juice of *Citrus junos* used as expectorant for cough and anti-skin aging medicine, body fruit of *Phellinus linteus* used as an anticancer, anti-skin aging drug (Yoon 1992; Huh 1981). Based on results of this report, similar data was found in our study.

In conclusion, C3-7 (Citron 3+*Phellinus linteus*), C3-15 (Citron 3+*Polygonum multiflorum*), C4-7 (Citron 4+*Phellinus linteus*), C4-8 (Citron 4+*Rehmannia glutinosa*) and C4-15 (Citron 4+*Polygonum multiflorum* Thunb.) showed strong cell proliferation activity on the CCD-986sk human fibroblast cell lines. While C4 complex groups were exhibited more strong cell proliferation activity than C3 complex groups, generally.

## ACKNOWLEDGEMENT

This study was supported by a grant from the Korea Ministry of Agriculture and forest.

## REFERENCES

- Braverman Irwin M. and Eileen Fonferko. 1982. Studies in cutaneous aging: II. The Microvasculature. Soc. Invest. Dermatol. 78:444-448.
- Chait A, M Mancini, AW February and B Lewis. 1972. Clinical and metabolic study of alcoholic hyperlipidemia. Lancet. 2:62-64.

- Choi HC and CH Oh. 1997. Evaluation of skin furrows in the aging process using an image analysis system. *Kor. J. Dermatol.* 35:292-302.
- Han KW, KB Myung, JH Hahm and HI Kook. 1987. The effect of sulfhydryl compounds on melanosomal morphology of epidermal melanocytes in UV-irradiated black mice. *Kor. J. Dermatol.* 25:553-561.
- Han KH, KH Cho, DY Noh, HC Eun and JI Youn. 1998. Histological changes in the skin with innate aging. *Kor. J. Dermatol.* 36:971-980.
- Huh J. 1981. *Young-In Bon. Dong Eu Bo Gam.* Hang-Sung Book Press, Seoul. 379-422.
- Kang S, XY Li and J John. 1996. *Voorhees. Pharmacology and molecular action of retinoids and vitamin D in skin.* *Soc. Invest. Dermatol.* 1(1):15-21.
- Kang BJ and KJ Park. 1997. Screening of antiviral activities of Korean medicinal herbs and traditional prescriptions against Herpes simplex virus type-1. *J. Kor. Soc. Virology.* 27(2):227-236.
- Kim YK, YK Park and HJ Kim. 1988. The effect of UV-B radiation on epidermal melanocytes of C57BL Mouse. *Kor. J. Dermatol.* 26:139-144.
- Kim YH, KS Ahn, DK Song and MB Wie. 1989. Effects of Panax Ginseng and Radix Astragali on age-related physiological alterations in rats. *J. K.O.M.S.* 10(2):26-46.
- Koo SW, DH Ban, YK Kim, SK Han and YK Park. 1998. The effects of ultraviolet radiation on aging and p53 expression in human skin. *Kor. J. Dermatol.* 36:224-235.
- Lavker, M Robert, Z Peishu and D Gang. 1987. Aged skin: A study by light, transmission electron, and scanning electron microscopy. *Soc. Invest. Dermatol.* 88:44-51.
- Lee OS, JE Hong, YK Cho and YS Lee. 1997. Studies on the synthesis of L-Ascorbic acid-3-aminopropane phosphoric acid diester and its applications. *J. Soc. Cos. Sci. Kor.* 23:97-109.
- Yoon KY. 1992. *Clinical Prescriptions of Korean traditional medicals.* Myung Bo Publishing, Seoul.

Manuscript Received: September 27, 2003

Revision Accepted: January 26, 2004

Responsible Editorial Member: Young Gyu Chai  
(Hanyang Univ.)