

## Korean Medicinal Herb Extracts Inhibit Melanin Formation in Clone M-3 Mouse Melanocyte Cell Lines

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**Abstract** - In order to search for anti-melanin formation agents from Korean medicinal herbs, we selected 21 Korean medicinal herbs, based on a review of Korean traditional medicine books and the recommendations of Korean traditional medical doctors. We tested for inhibition effect of melanin pigmentation of Clone M-3 mouse melanocyte cell lines when we treated the extracts of 21 medicinal herbs in the mouse melanocyte cell lines, respectively. Among 21 medicinal herb extracts, 5 extracts showed a inhibition effect of melanin formation. The sample *Phaseolus radiatus* L, *Cordyceps militaris*, *Pinellia ternata*, *Phellinus linteus* and *Citrus junos* Tanaka showed a significantly little formation of melanin pigments compared with control groups. Especially extract of *Citrus junos* Tanaka was more potent than the others. These results suggest that extract of Korean *Citrus junos* may represents an excellent candidate for inhibition of melanin pigmentation at *in vitro* level.

**Key words** : *Citrus junos* Tanaka, Medicinal herbs, clone M-3 mouse melanocyte cell lines, inhibition effect of melanocyte

### 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 darkend and thickened. 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 a 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 number. Kim (1988) showed the effect of UV-B radiation on epidermal melanocytes of C57BL mouse. And Han (1987) reported on the effect

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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 and melanin formation. 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 melanin formation 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 Korean medicinal herb extracts protect melanin formation at the *in vitro* level, thereby resulting in protection from increased roughness, skin darkness and mottling (Lavker *et al.* 1987).

## MATERIALS AND METHODS

The Clone M-3 cell line (mouse melanocyte, KCBL-10053.1) was obtained from Korean Cell Line Bank (KCLB; Seoul, Korea). Clone M-3 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.

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 November (ripened for four months) of 2003. Mellowed citrons 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, France) and used experiment.

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). All Samples were stored at 4°C. Each extract (100 μL, added medicinal herb at the concentration of 200 mg mL<sup>-1</sup>) was diluted sequentially in two-fold steps with PBS and then added to each well of 6-well culture plate containing 1.2 × 10<sup>6</sup> Clone M-3 mouse melanocyte cell lines per 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 4 days and the amount of

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

No.	Family and species	Voucher no.	Vernacular name(s)	Efficacy after treatment
1	Labiatae <i>Agastache rugosa</i>	1-1k	KwakYang	Aerial part used to dispel summer heat and dampness
2	Leguminosae <i>Phaseolus radiatus</i> L.	1-2k	NokDu	Fruits used as cutaneous disease
3	Umbelliferae <i>Angelica gigas</i> Nakai	1-3k	TangGwi	Root juice used to nourish the blood and to invigorate the blood circulation
4	Cordycepitateae <i>Cordyceps militaris</i>	1-4k	DongChungHaCho	Fruit body used as an antidote and anticancer drug
5	Labiatae <i>Mentha haplocalyx</i> Briq.	1-5k	PaKa	Aerial part used for affection due to wind and heart with fever, headache, stuffy nose and sore throat
6	Araceae <i>Pinellia ternata</i>	1-6k	PanHa	Tuberous roots used for the treatment of emesis and diarrhea
7	Hymenochaetaceae <i>Phellinus linteus</i>	1-7k	SangHwang	Fruit body used as an anticancer drug and antidote
8	Scrophulariaceae <i>Rehmannia glutinosa</i>	1-8k	SaengGiHwang	Tuberous roots used for the treatment of thirst, exanthesis and bleeding
9	Ranunculaceae <i>Cimicifuga heracleifolia</i> Kom.	1-9k	SeungMa	Root juice used as an antipyretic and antidote
10	Betulaceae <i>Alnus japonica</i>	1-10k	OhRi	Bark used as an antipyretic and styptic
11	Ranunculaceae <i>Paeonia lactiflora</i> Pall.	1-11k	ChagYak	Root juice used to nourish the blood and pain-relieving agent for headache
12	Compositae <i>Xanthium strumarium</i> L.	1-12k	ChangYiJa	Fruits used to treat cough and fever
13	Compositae <i>Atractylodes japonica</i> Koidz.	1-13k	ChangChul	Rhizome juice used for treating dyspepsia due to accumulation of dampness
14	Rubiaceae <i>Gardenia jasminoides</i> Ellis.	1-14k	ChiCha	Ripe fruit juice used as antipyretic and sedative for fever with fidgetiness
15	Polygonaceae <i>Polygonum multiflorum</i> Thunb.	1-15k	HaSuOh	Tuberous roots used for robustness
16	Rosaceae <i>Prunus armeniaca</i> L.	1-16k	HaengIn	Seeds used to relieve cough
17	Cyperaceae <i>Cyperus rotundus</i> L.	1-17k	HyangPuJa	Tuberous roots used to treat headache and stomachache
18	Scrophulariaceae <i>Scrophularia buergeriana</i> Miq.	1-18k	HyonSam	Root juice used to treat sore throat and swelling
19	Labiatae <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 <i>Coptis japonica</i> Makino	1-20k	HwangRyon	Rhizome juice used as antiphlogistic for various infections
21	Rutaceae <i>Citrus junos</i> Tanaka	1-21k	YuJa	Ripe fruit juice used as anti-cough and anti-aging

melanin pigments in each well was estimated using spectrophotometer at 475 nm wavelength. The same test was done six times and 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

Twenty one Korean medicinal herb extracts were screened for their activated effect on Clone M-3 mouse melanocyte cell lines by measure of OD values using spectrophotometer at 475 nm wavelength. Diluted PBS and 0.8% methanol was used normal control group.

**Table 2.** Estimation of melanin pigmentation on the melanocyte treated with Korean medicinal herb extracts

Test groups	O.D. at 475 nm
PBS	0.178±0.008
Methanol	0.187±0.006
2 (NokDu)	0.169±0.002
4 (DongChungHaCho)	0.127±0.007*
6 (PanHa)	0.175±0.010
7 (SangHwang)	0.155±0.006*
21 (YuJa)	0.101±0.008*

Each arabic numeral indicates Korean herb extracts as shown in Table 1. All values are mean ± standard deviation. \* values are significantly different from the control at  $P < 0.1$  as determined by student t-test. PBS is normal control group. Methanol (0.8%) is negative control group.

The aim of this study was to investigate the anti-melanin formation effects of Korean citrus junos and medicinal herbs at *in vitro* level. The effect of five Korean medicinal herbs on Clone M-3 mouse melanocyte cell lines was clearly powerful as shown in Table 2. The extracts of *Phaseolus radiatus* L, *Cordyceps militaris*, *Pinellia ternata*, *Phellinus linteus* and *Citrus junos* Tanaka showed strong cell proliferation activity compared with the normal control group. The OD values of *Phaseolus radiatus*, *Cordyceps militaris*, *Pinellia ternata*, *Phellinus linteus* and *Citrus junos* Tanaka showed 0.169±0.002, 0.127±0.007, 0.175±0.010, 0.155±0.006 and 0.101±0.008, respectively. The extract of *Citrus junos* Tanaka showed very strong anti-melanin formation effects compared with other groups. The OD value of *Citrus junos* Tanaka was lower 76 percent than normal control group (PBS). Based on reports (Lavker *et al.* 1987; Choi 1997; Han 1998) 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 and skin darkness, a number of drugs have been developed to renature the skin collagen and elastin and to protect darkness of skin horny layer. However, little work has been done in developing natural materials to prevent skin darkness and skin-aging. In Korea, many Korean medicinal plants and traditional prescriptions have a long history of clinical application. Indeed, they are utilized as anti-skin darkness agents without major side effects. We, therefore, focused on the extracts of these plants as candidate for anti-skin darkness

agents. In this study, the major finding is that 5 extracts of Korean medicinal plants exhibiting melanin formation inhibition activity and that especially extracts of *Citrus junos* Tanaka exhibiting significant melanin formation inhibition activity. This report suggests that Korean citrus junos may represent an alternative therapeutic agent to assist in the prevention and treatment of skin-darkness.

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