



Original Article

## The Experimental Study on Inhibitory Effects of Wild Ginseng Pharmacopuncture Solution on Melanin Biosynthesis

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### ABSTRACT

#### Article history:

Submitted: July 24, 2018

Revised: August 10, 2018

Accepted: August 17, 2018

#### Keywords:

ginseng, melanin, B16 melanoma, pharmacopuncture, traditional medicine

**Background:** The purpose of this study was to investigate the effects of wild ginseng pharmacopuncture on melanin production in B16/F10 murine melanoma cells.

**Methods:** To determine the effect of wild ginseng pharmacopuncture solution on B16/F10 cells, cytotoxicity was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) method. To observe B16/F10 cell growth, death, and morphological changes, Trypan blue solution was used. The Hosoi method was used to investigate the effect of wild ginseng pharmacopuncture solution on melanin production. The Martinez-Esparza method was used to investigate the effect of wild ginseng pharmacopuncture solution on tyrosinase activity. To determine the pathway involved in the melanogenesis in cells exposed to wild ginseng pharmacopuncture solution, a cell-free tyrosinase was used.

**Results:** Following treatment with 200  $\mu$ L of wild ginseng solution, the cell survival rate was  $76.32 \pm 2.45\%$  which significantly decreased with higher concentrations ( $\mu$ L) of wild ginseng (up to 200  $\mu$ L). When 100  $\mu$ L of wild ginseng was used, the cell survival rate was  $89.95 \pm 2.07\%$ . No morphological changes or abnormalities were observed in the B16/F10 murine melanoma cells as observed in the Trypan blue test. Melanin production was significantly reduced to  $72.17 \pm 3.74\%$  at 100  $\mu$ L. Using 100  $\mu$ L of wild ginseng solution, tyrosinase activity was significantly decreased to  $80.15 \pm 1.05\%$ . Wild ginseng pharmacopuncture solution reduced melanin production both directly and indirectly.

**Conclusion:** This study suggests that wild ginseng pharmacopuncture solution may be effective in inhibiting melanin production. Further studies are needed to determine safe and effective clinical applications.

<https://doi.org/10.13045/jar.2018.00171>  
pISSN 2586-288X eISSN 2586-2898

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### Introduction

Human skin color depends on the amount of melanin contained within each epidermal unit. Melanocytes function in association with keratinocytes and Langerhans cells. The functional units associated with melanocytes, keratinocytes, and Langerhans cells are called epidermal melanin units [1]. Melanin is produced by a tyrosinase enzymatic reaction in melanocytes. The resulting melanin protects the skin from external stimuli, such as ultraviolet (UV) rays [2]. Melanin causes pigmentation. This prevents photoaging caused by UV rays [3], however, the precursor of melanin promotes apoptosis and has a negative function in terms of cosmetic appearance [4,5]. Therefore, studies have been performed to investigate ways to prevent or reduce hyperpigmentation in the skin. Cosmetics and traditional Korean medicines containing hydroquinone, arbutin, kojic acid, vitamin C, and others are sold

based on their effects on hyperpigmentation [6-8]. However, research is ongoing to discover raw materials that exhibit better properties for preventing hyperpigmentation effects. Recently, there have been studies published on the effects of Pueraria [9], Siberian Sibiricum [10], and Hyssopus officinalis [11] on melanogenesis.

Wild ginseng is grown naturally in the wild and is used to supplement energy in Korean medicine. It is a typical drug to treat physical decline, fatigue, and burnout [12]. Wild ginseng has almost the same active ingredient composition as ginseng and high active ingredient content, so it exhibits excellent effects for symptom treatment [13].

Wild ginseng pharmacopuncture is a traditional Korean medication. The wild ginseng used for pharmacopuncture solution is extracted by distillation and injected directly into acupuncture points, pressure points, tender points, muscles, and blood vessels.

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It is an accepted acupuncture treatment in Korean medicine, and the acupuncture and medicinal effects of wild ginseng can be used in parallel [14].

In Korean medicine, hyperpigmentation is known to occur more in people with a lack of physiologically active substances, energy incompatibility, and emotional stress [15]. Therefore, there has been research to reduce pigmentation mainly through treatment with herbal medicine [16-18]. In previous studies, the whitening effect using hominis placenta has been studied [19]. To date there have been very few studies on the effects of wild ginseng pharmacopuncture solution on reducing dermal hyperpigmentation.

Therefore, we performed this study to investigate the effect of wild ginseng pharmacopuncture solution on potentially inhibiting hyperpigmentation of the skin, by measuring the changes in cell number, the amount of melanin, cell morphology, tyrosinase activity, and cell-free tyrosinase in B16/F10 murine melanoma cells.

## Materials and Methods

### Materials

Sanyangsansam wild ginseng pharmacopuncture solution was used in the experiments (Korean Pharmacopuncture Research Institute, Wonju, Korea).

### Cell culture

B16/F10 murine melanoma cells (Korean Cell Line Bank) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and penicillin-streptomycin at 37°C, 5% CO<sub>2</sub>.

### Measuring cytotoxicity

The B16/F10 melanoma cells were cultured at 37°C, 5% CO<sub>2</sub> using serum-free DMEM medium. Cell viability was measured using Mosmann's method [20].  $1 \times 10^5$  B16/F10 melanoma cells were placed in each well of a 96-well plate and cultured for 24 hours. The cells were treated with wild ginseng pharmacopuncture solution at different concentrations ( $\mu$ L) and cultured for 72 hours. After incubation, 0.05% 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) solution was added and cultured at 37°C for 2 hours. The supernatant was removed, 1 mL of dimethyl sulfoxide (DMSO) was added to the formazan precipitate, and it was allowed to stand at room temperature for approximately 15 minutes. The absorbance was measured at a wavelength of 540 nm, and cell viability was calculated.

### Trypan blue test

B16/F10 melanoma cells were cultivated at  $5 \times 10^5$ /well in a 6-well plate. Cells were cultured for 24 hours at 37°C, 5% CO<sub>2</sub>. Cells were separated from each well using trypsin. The separated cells were washed twice with phosphate-buffered saline (PBS). A total of 2 mL of 0.4% (w/v) trypan blue solution was added to each well. A hemocytometer was used to count the number of living cells (cells without trypan blue staining are living).

### Measuring melanin amount

The amount of melanin was measured by applying the Hosoi [21] method. The cultured cells were centrifuged at 5,000x g, for

5 minutes. A total of 200  $\mu$ L of 1 N NaOH solution was added, to which 10% DMSO was also added. Following 1 hour at 80°C the absorbance was measured at 405 nm. The amount of melanin contained in the sample was calculated using a melanin standard curve.

### Measuring tyrosinase activity

The tyrosinase activity was measured by the Martinez-Esparza [22] method. The cells in each well were centrifuged (5,000x g, 5 minutes) to precipitate the cells. A total of 100 mL of lysis buffer was added to the cell pellet and placed in ice. The supernatant was separated by centrifugation (5,000x g, 5 minutes) again. A total of 100 mL of 100 mM sodium phosphate was added to 100  $\mu$ L of the sample. It was reacted at 37°C for 5 minutes. A total of 50  $\mu$ L of 100 mM catechol solution was added. Absorbance was measured at 405 nm.

### Cell-free tyrosinase

The tyrosinase activity was determined by measuring the absorbance at 475 nm using 3,4 Dihydroxy-L-phenylalanine (L-DOPA) as a substrate. A total of 160  $\mu$ L of 0.1 mM potassium phosphate buffer and 20  $\mu$ L of 0.01% L-DOPA were added, and 10  $\mu$ L of each sample and enzyme solution (mushroom tyrosinase) were added, followed by reaction at 37°C for 1 hour. After the reaction, the absorbance at 475 nm was measured and the tyrosinase activity was calculated as a percentage based on the absorbance of the control group.

### Statistical analysis

The results were expressed using averages  $\pm$  SD with the SPSS Windows program (Ver. 21.0). Statistical significance was confirmed using the Student's t test. The level of significance was  $p < 0.05$ .

## Results

### Measuring cytotoxicity

Cells were treated with varying concentrations of 0, 10, 20, 50, 100, and 200  $\mu$ L/mL of cell suspension to determine the optimum treatment concentration ( $\mu$ L) of wild ginseng pharmacopuncture solution on B16/F10 melanoma cells. After 72 hours, cell viability was examined by MTT assay. The cell viabilities were  $100.00 \pm 0.14\%$ ,  $98.49 \pm 1.75\%$ ,  $97.87 \pm 1.84\%$ ,  $94.29 \pm 1.93\%$ ,  $89.95 \pm 2.07\%$ , and  $76.32 \pm 2.45\%$  of the negative control at the wild ginseng pharmacopuncture solution concentrations of 0, 10, 20, 50, 100, and 200  $\mu$ L/mL of cell suspension, respectively.

The MTT assay showed a significant decrease in cell viability using 200  $\mu$ L wild ginseng pharmacopuncture solution/mL of cell culture (Fig. 1).

### Trypan blue test

Trypan blue uptake was used to examine the number of living and dead cells. With higher concentrations of wild ginseng the number of living cells decreased significantly. The cell viabilities were  $100.00 \pm 0.22\%$ ,  $98.14 \pm 2.16\%$ ,  $95.17 \pm 1.78\%$ ,  $91.67 \pm 2.97\%$ ,  $89.74 \pm 1.74\%$ , and  $84.11 \pm 3.78\%$  at the wild ginseng pharmacopuncture solution concentrations of 0, 10, 20, 50, 100, and 200  $\mu$ L/mL, respectively. The number of viable cells decreased in a dose-dependent manner. These results showed similar results

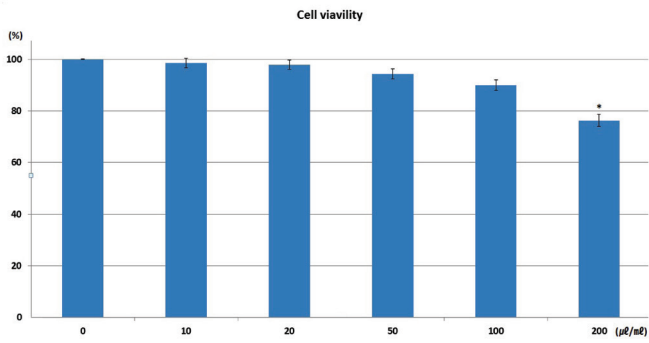


Fig. 1. Effect of wild ginseng pharmacopuncture solution on cell viability. The cell cytotoxicity of the B16/F10 murine melanoma cells was measured by MTT assay. Results were expressed as % of untreated control. \* $p < 0.05$ .

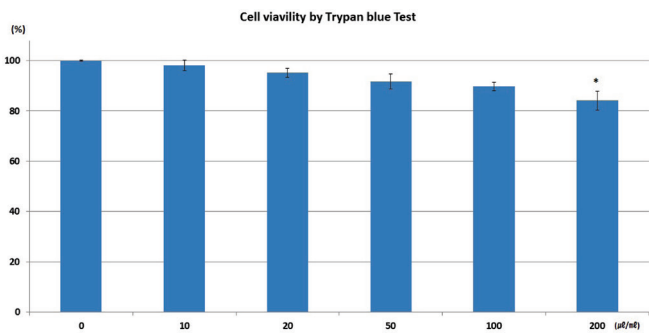


Fig. 2. Effect of wild ginseng pharmacopuncture solution on cell viability by trypan blue test. The cell viability of the B16/F10 murine melanoma cells was measured by trypan blue test. Results were expressed as % of untreated control. \* $p < 0.05$ .

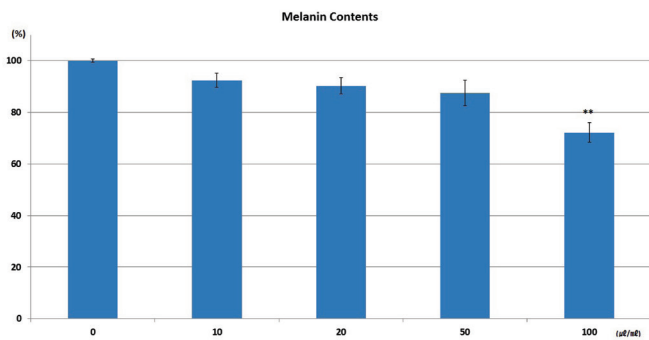


Fig. 3. Effect of wild ginseng pharmacopuncture solution on melanin content. The melanin content of the B16/F10 murine melanoma cells was measured by Hosoi method. Results were expressed as % of untreated control containing no ginseng pharmacopuncture solution. \*\* $p < 0.01$ .

to the MTT measurements. The morphological abnormality of the cells was not examined (Fig. 2).

### Measuring melanin amount

To investigate the effect of wild ginseng pharmacopuncture solution on the melanin synthesis in B16/F10 melanoma cells, the total melanin levels were measured after culturing wild ginseng at 0, 10, 20, 50, and 100 µL/mL for 3 days. The results

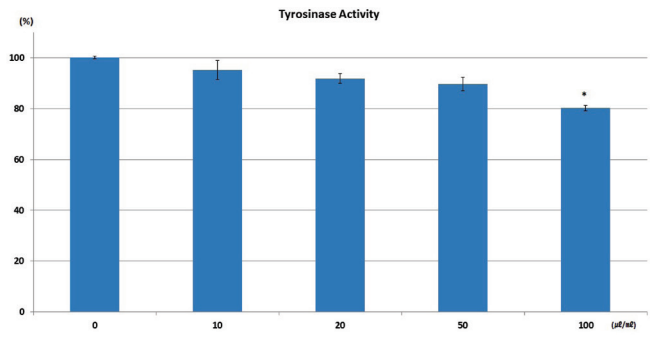


Fig. 4. Effect of wild ginseng pharmacopuncture solution on cell tyrosinase activity. The Tyrosinase activity of the B16/F10 murine melanoma cells was measured by Martines Esparza method. Results were expressed as % of untreated control. \* $p < 0.05$ .

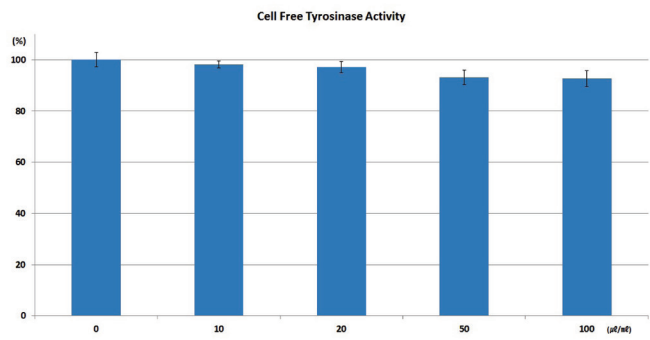


Fig. 5. Effect of wild ginseng pharmacopuncture solution on cell free tyrosinase activity. The Tyrosinase activity on cell free was measured by Mushroom tyrosinase. Results were expressed as % of control.

showed that melanin production in B16/F10 melanoma cells after treatment with wild ginseng pharmacopuncture solution was  $100.00 \pm 0.59\%$ ,  $92.44 \pm 2.72\%$ ,  $90.26 \pm 3.17\%$ ,  $87.47 \pm 4.91\%$ , and  $72.17 \pm 3.74\%$  compared with the negative untreated control cells after treatment with 0, 10, 20, 50, and 100 µL/mL of wild ginseng pharmacopuncture solution, respectively. Wild ginseng pharmacopuncture solution significantly decreased melanin synthesis at 100 µL/mL (Fig. 3).

### Measuring tyrosinase activity

The effect of wild ginseng pharmacopuncture solution on the tyrosinase activity in B16/F10 melanoma cells was  $100.00 \pm 0.57\%$ ,  $95.11 \pm 3.69\%$ ,  $91.84 \pm 1.95\%$ ,  $89.65 \pm 2.74\%$ , and  $80.15 \pm 1.05\%$  compared with the untreated control cells treated with 0, 10, 20, 50, and 100 µL/mL wild ginseng pharmacopuncture solution, respectively. Wild ginseng pharmacopuncture solution significant decreased tyrosinase activity at 100 µL/mL (Fig. 4).

### Cell free tyrosinase activity

The inhibition of tyrosinase activity by mushroom tyrosinase can be regulated by controlling the oxidation and reduction of tyrosinase by changing the state of copper ions at an active tyrosinase site. Therefore, the inhibition of in vitro tyrosinase activity by mushroom tyrosinase provides a useful experimental assessment. The effect of wild ginseng pharmacopuncture solution

on the tyrosinase activity of B16/F10 melanoma cells was  $100.00 \pm 2.81\%$ ,  $98.16 \pm 1.26\%$ ,  $97.12 \pm 2.18\%$ ,  $83.15 \pm 2.75\%$ , and  $92.66 \pm 3.17\%$  compared with untreated controls when incubated with 0, 10, 20, 50, and 100  $\mu\text{L}/\text{mL}$  of wild ginseng pharmacopuncture solution, respectively. However, these results were not statistically significant. In this experiment, the direct tyrosinase-inhibitory effect of wild ginseng pharmacopuncture solution was tested using mushroom tyrosinase. As a result, wild ginseng pharmacopuncture solution directly inhibited concentration-dependent activity of the mushroom tyrosinase enzyme (Fig. 5).

## Discussion

Melanin, hemoglobin, and carotene are the 3 factors that determine skin color. The most important of these is melanin. The main function of melanin is removing reactive oxygen species or free radicals from the skin, and protecting skin by blocking UV transmission [1].

Melanin is a polymeric substance of phenols released by melanocytes, which are distributed in the skin, hair follicles, eyes, ears, and cerebrum in the human body. Melanin cells, fibroblasts, keratinocytes, and inflammatory cells are activated due to external stresses such as UV rays, and pigmentation is controlled by prostaglandin, various cytokines, and changes in  $\alpha$ -Melanocyte-stimulating hormone ( $\alpha$ -MSH). In particular, several factors, such as  $\alpha$ -MSH, adrenocorticotropic hormone, and  $\beta$ -endorphin secreted from keratinocytes, affect melanocyte growth, morphology, and differentiation. Ultimately, melanin is transmitted to keratinocytes through dendrites, thereby inhibiting cytotoxicity from the cells [15].

Tyrosinase is an important enzyme in the melanin synthesis process. It is involved in the oxidation of tyrosine, an in vivo amino acid, to L-DOPA. In eumelanogenesis, which produces brownish eumelanin, DOPA quinone is converted to DOPA chrome, and DOPA chrome becomes 5,6- di hydroxy indole-2-carboxylic acid (DHICA) due to tyrosinase-related protein-2, and DHICA eumelanin due to tyrosinase-related protein-1. On the other hand, DOPA chrome is converted to indole-5, 6-quinone by tyrosinase oxidase and DHI eumelanin is finally produced by reduction and decarboxylation. When the concentrations of sulfur-containing amino acids, such as cysteine and glutathione, are high, DOPA quinone forms cysteinyl DOPA and is converted into pheomelanin after several transitions. In general, melanin refers to the eumelanin produced by eumelanogenesis. Melanin produced from the melanin body is transferred to the keratinized cells of the epidermis through the dendritic processes and transferred to the surface by turn-over and eventually separated and eliminated [5].

Skin color and pigmentation are determined by the amount and distribution of melanin in keratinocytes. It is important to inhibit the production of melanin by inhibiting tyrosinase activity. To date, known inhibitors of tyrosinase activity include hydroquinone, kojic acid, and arbutin. They inhibit tyrosine oxidation and melanin production, including vitamin C and glutathione. In addition, salicylic acid has the effect of promoting the removal of the stratum corneum. Research studies on the effects of retinoic acid, alpha hydroxy acid, UV barrier material, cytotoxic inhibition, and active oxygen removal have been performed [6-8].

In this study, we first examined the cell viability following exposure to wild ginseng pharmacopuncture solution and found that the cell viability was reduced to 76.32% up to concentrations of 200  $\mu\text{L}/\text{mL}$ . These results show similar trends to the trypan blue measurements. There was also statistically significant cell loss at the 200  $\mu\text{L}/\text{mL}$  concentration in the trypan test.

Wild ginseng pharmacopuncture solution reduced tyrosinase

activity to 89.65% and 80.15% at concentrations of 50 and 100  $\mu\text{L}/\text{mL}$ , respectively. Thus, wild ginseng pharmacopuncture solution concentration decreased tyrosinase activity in B16/F10 melanoma cells. In the total melanin test, melanin synthesis was reduced to 87.47% and 72.17% compared with the control group at 50 and 100  $\mu\text{L}/\text{mL}$ , respectively, which was similar to that of tyrosinase activity. In previous experiments, tyrosinase activity and total melanin level was very closely related [5].

To examine whether wild ginseng pharmacopuncture solution directly inhibits tyrosinase activity, the key enzyme of melanogenesis, mushroom tyrosinase was not directly inhibited by mushroom tyrosinase enzyme activity in a cell-free system. As a result, it was found that wild ginseng pharmacopuncture solution also inhibits tyrosinase activity directly through the signal transduction pathway of B16/F10 melanoma cells.

In conclusion, it was found that wild ginseng pharmacopuncture solution inhibits melanin production in B16/F10 melanoma cells and may play a role in as a drug substance for the treatment of skin blemishes and freckles, and for cosmetic skin whitening. Further research should be conducted to investigate its possibility for stable and efficient clinical use.

## Conclusion

1. The cell viability of wild ginseng pharmacopuncture solution was decreased to 76% at a concentration of 200  $\mu\text{L}/\text{mL}$ .
2. Wild ginseng pharmacopuncture solution did not cause morphological changes in the B16/F10 cells.
3. Wild ginseng pharmacopuncture solution resulted in a statistically significant decrease in the total amount of melanin produced in the B16/F10 cells at 100  $\mu\text{L}/\text{mL}$ .
4. Wild ginseng pharmacopuncture solution produced a statistically significant decrease in tyrosinase activity in the B16/F10 cells at 100  $\mu\text{L}/\text{mL}$ .
5. Wild ginseng pharmacopuncture solution directly decreased tyrosinase activity
6. Wild ginseng pharmacopuncture solution directly and indirectly inhibited the activity of the mushroom tyrosinase enzyme. Direct inhibition pathways were statistically significant.
7. Wild ginseng pharmacopuncture solution may reduce melanin synthesis by inhibiting the tyrosinase biosynthesis of melanocytes, thus decreasing the expression of tyrosinase which is involved in melanin production.

## Conflicts of Interest

The authors have no conflicts of interest to declare.

## Acknowledgments

This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIP; Ministry of Science, ICT & Future Planning) (No. NRF-2017R1C1B5017799).

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