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Melanin Biosynthesis Inhibition Effects of Ginsenoside Rb2 Isolated from *Panax ginseng* Berry

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Ginsenoside Rb2 (Gin-Rb2) was purified from the fruit extract of *Panax ginseng*. Its chemical structure was measured by spectroscopic analysis, including HR-FAB-MS, ^{1}H-NMR, and IR spectroscopy. Gin-Rb2 decreased potent melanogenesis in melan-a cells, with 23.4% at 80 μM without cytotoxicity. Gin-Rb2 also decreased tyrosinase and MITF protein expression in melan-a cells. Furthermore, Gin-Rb2 presented inhibition of the body pigmentation in the zebrafish *in vivo* system and reduced melanin contents and tyrosinase activity. These results show that Gin-Rb2 isolated from *P. ginseng* may be an effective skin-whitening agent *via* the *in vitro* and *in vivo* systems.

Keywords: Ginsenoside Rb2, *Panax ginseng*, melanin biosynthesis, melan-a cell, zebrafish

Much effort has focused on developing skin-depigmenting products for people with unwanted pigments in the cosmetic field. Melanin acts as a defense mechanism against ultraviolet (UV) light, but unusual accumulation of melanin can cause hyperpigmentation in the epidermis. Melanin overexpression induces serious esthetic problems, such as freckles, solar lentigo, melasma, ephelide, and other forms on the human body [4]. Moreover, UV rays, the main cause of melanin synthesis, have been increasing owing to recent environmental pollutions and change of living style; accordingly, people are interested in new skin-whitening agents [30]. Several of the known melanogenesis inhibitors have already been the focus of studies, such as arbutin and kojic acid, and are currently being utilized as cosmetic additives [7]. However, it is clearly necessary to find more safer and effective depigmenting agents because of the potential carcinogenic side effect of kojic acid [12]. Owing to various safety concerns and the lower efficacy of commercially available skin-whitening agents, the isolation of new compounds from natural products that prevent pigment disturbances has attracted much interest.

Panax ginseng C.A. Meyer has been used in traditional folk medicine and food in Asian countries. Usually, most of the *P. ginseng* studies have focused on the components of the 4 to 6 years of root. Besides the *P. ginseng* root, the fruits also have pharmacological effects such as antidiabetic [23, 25], anticancer [21, 26], antioxidant [18, 24], antiaging [9], and antiallergic effects [1]. Furthermore, ginsenoside Rb2 (Gin-Rb2) from *P. ginseng* was reported to have various biological activities, including radioprotective, antitumor, antivirus, and antidiabetic effects [15–17, 28]. However, the whitening activity of Gin-Rb2 has not previously been reported. Therefore, we have analyzed the anti-melanogenesis through *in vitro* and *in vivo* systems with Gin-Rb2 isolated

from the *P. ginseng* berry.

In order to search for pharmacologically active components from the *P. ginseng* berry, fresh ginseng fruits (5 kg) were extracted three times with 90% MeOH (50 L) for 1 day at 25°C. The extracts were filtered through filter paper and the filtrate was concentrated by evaporation. The dried extract (552 g) was diluted with H_2O (3 L) and extracted with EtOAc (3 L × 3). The remaining H_2O layer was extracted three times with *n*-BuOH (2.8 L × 3). Three solvent extracts were obtained as EtOAc (39 g), *n*-BuOH (166 g), and H_2O (306 g). The BuOH extract (PGFB, 166 g) was chromatographed on a column of Si gel with $CHCl_3$:MeOH:H_2O mixtures (step 1, 60 L of 65:35:10; step 2, 30 L of 6:4:1) to yield 13 fractions (PGFB1–PGFB13). The active fraction 10 (8 g) was purified by Si gel column (φ 8 × 15 cm) with a $CHCl_3$:MeOH:H_2O (65:35:10) eluent (5 L) to obtain 10 fractions (PGFB10-1–PGFB10-10). PGFB10-6 (3 g, Ve/Vt = 0.62–0.72) was further fractionated over an octadecyl silica gel (ODS) column (φ 4 × 14 cm, MeOH-H_2O = 3:2, 20 L) to afford 8 additional subfractions (PGFB10-6-1–PGFB10-6-8) including Gin-Rb2 (PGFB10-6-3, 100 mg, Ve/Vt = 0.21–0.32, TLC Rf = 0.32 (RP-18 F254S, MeOH-H_2O = 3:1), Rf = 0.46 (Kieselgel 60 F254, $CHCl_3$-MeOH-H_2O = 65:35:10)).

Gin-Rb2 was obtained as white powder from methanol, exhibiting a melting point value maximum at 183°C. The molecular ion of Gin-Rb2 was observed at m/z 1101 $[M+Na]^+$ in the positive FAB/MS. According to reported NMR and MS data by Zhao *et al.* [31], the isolated compound was identified as Gin-Rb2 (Fig. 1A) [31].

Melan-a cells have provided an excellent parallel non-tumor cell line for testing the malignancy of melanomas, and its phenotype is similar to mice with primary melanocytes [2]. To determine the inhibition of melanogenesis activity of Gin-Rb2, it was treated into melan-a cells with 0 to 160 μM for 3 days and cytotoxic effects were determinate by the CCK-8 kit. The Gin-Rb2 showed no cytotoxic effect

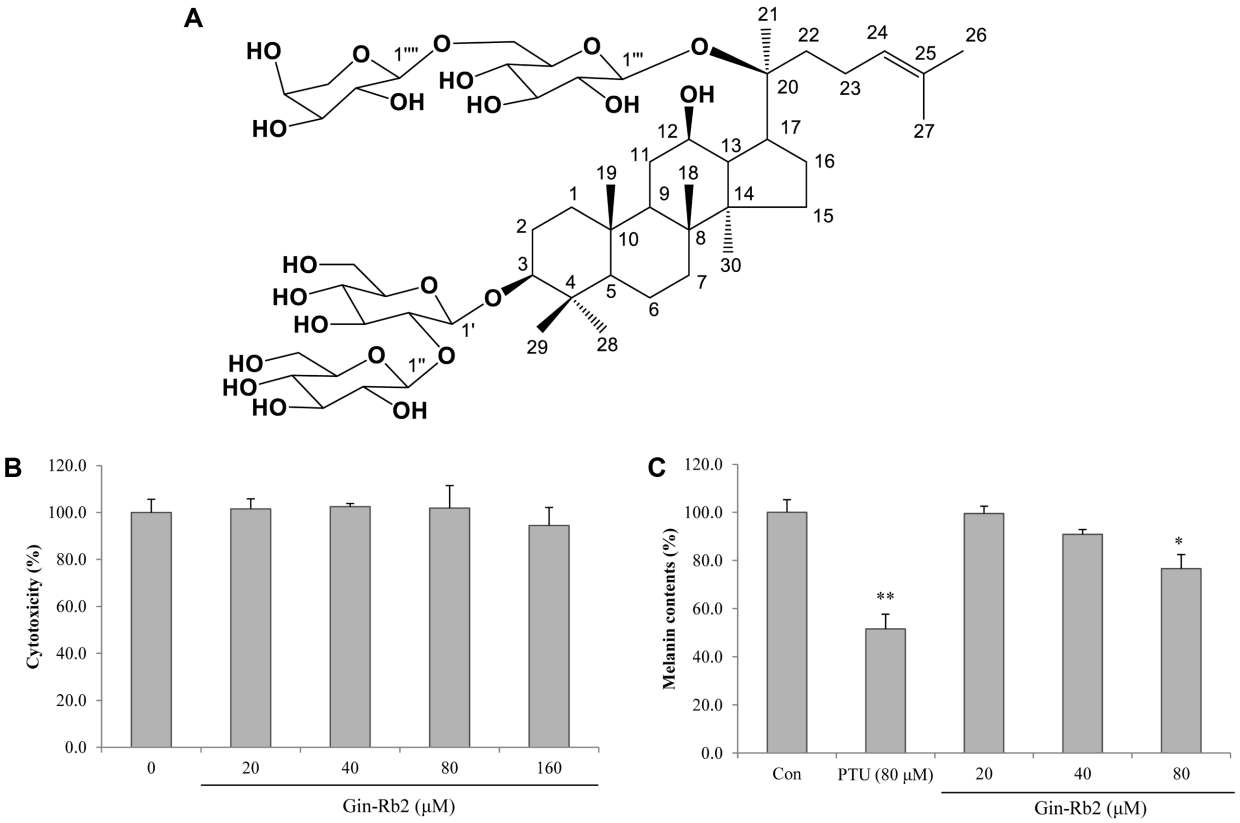

Fig. 1. Effects of ginsenoside Rb2 (Gin-Rb2) on melanogenesis in melan-a cells.

(**A**) The structure of ginsenoside Rb2. (**B**) Effect of cell cytotoxicity was measured with triplicate experiments. The cells were cultured with 0–160 μM of Gin-Rb2 for 3 days, and cell cytotoxicity was determined by a CCK-8 cell counting kit. (**C**) Inhibition of melanin synthesis was measured with triplicate experiment. The cells were cultured with 0–80 μM of Gin-Rb2 for 3 days. Each value is expressed the mean ± SD of triplicate determinations. *$p < 0.05$, **$p < 0.01$ versus control group.

at all tested concentrations in the melan-a cells (Fig. 1B). Fig. 1C shows that melanin levels were decreased in a dose-dependent manner by Gin-Rb2-treated melan-a cells. Notably, Gin-Rb2 indicated the significantly highest melanin inhibitory activity as 33.4% at 80 μM concentration. Reportedly, cinnamic acid, a depigmentation agent found mainly in *P. ginseng*, showed 29% decrease of melanin contents and reduced tyrosinase activity at 500 μM in melan-a cells [13]. Recently, we reported that ginsenoside Rh6, vina-ginsenoside R4, and vina-ginsenoside R13 isolated from *P. ginseng* were shown to have anti-melanogenesis activity [14]. On the other hand, the extract of Radix Ginseng did not exhibit any significant inhibition of melanin synthesis [10].

Through an enzymatic cascade, such as tyrosinase, the enzyme catalyzing the rate-limiting step in melanin biosynthesis, and microphthalmia-associated transcription factor (MITF) level regulated melanogenesis [3, 8]. MITF also plays a pivotal role in effectively activating the tyrosinase melanogenic genes *in vitro* [27]. There was marked reduction of tyrosinase and MITF protein expression with Gin-Rb2 treatment at 80 μM compared with the vehicle treatment (Fig. 2).

Traditionally, zebrafish has been used in the fields of biochemical studies. In particular, the use of embryos is receiving increasing attention since they are considered as replacement methods for animal experiments [19]. Melanin pigments can be observed on the zebrafish surface without any complicated experimental procedures [5]. Therefore, we used the zebrafish *in vivo* model to study the Gin-Rb2 anti-melanogenic effect. L-Tyrosine, 12-*o*-tetradecanoylphorbol-13-acetate, and 1-phenyl-2-thiourea (PTU) as positive controls have been widely used in zebrafish study [6]. The number of melanin pigment spots in the zebrafish body treated with Gin-Rb2 was reduced at the 80 μM concentration (Fig. 3A). Moreover, the tyrosinase activity and total melanin content on the zebrafish were significantly decreased after being treated with Gin-Rb2 at 80 μM (Fig. 3B).

In a recent report, melanin synthesis was inhibited by ginsenoside Rb1 on α-MSH (α-melanocyte stimulating hormone)-induced B16 melanoma cells and shown in a dose-dependent manner [20]. Lee *et al*. [14] purified minor ginsenosides from *P. ginseng*, which reduced body pigmentation on the zebrafish *in vivo* system. However, the Gin-Rb2 in this study has not been reported elsewhere. The

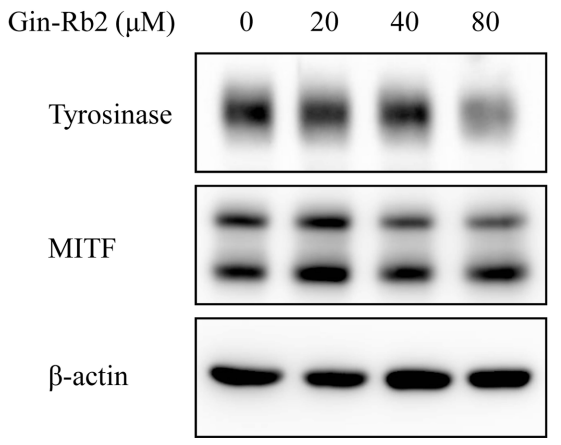

Fig. 2. Effects of ginsenoside Rb2 (Gin-Rb2) on tyrosinase and MITF protein expression in melan-a cells.

Melan-a cells were treated with 0–80 μM of Gin-Rb2 for 24 h. Total cell lysates were then prepared and subjected to western blotting using tyrosinase and MITF.

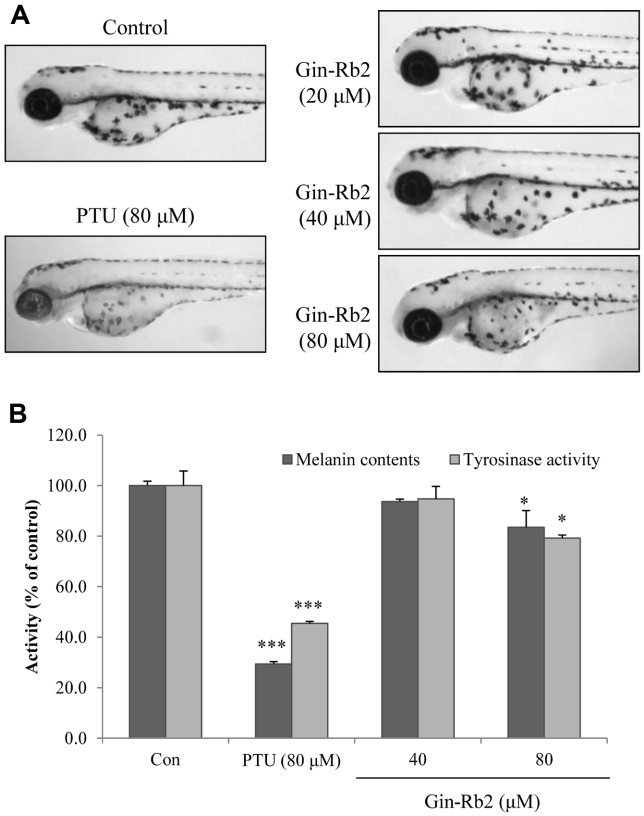

Fig. 3. Effects of ginsenoside Rb2 (Gin-Rb2) on melanin synthesis in zebrafish embryos.

Synchronized embryos were treated with the indicated concentrations of PTU and ginsenoside Rb2. Gin-Rb2 was dissolved in DMSO (0.1%) and then added to the embryo medium. (**A**) The change of phenotype on zebrafish was assessed using a microscope. (**B**) Total melanin content and tyrosinase activity were quantified using a spectrometer. Results shown are the mean of three independent experiments ± SD. $^{*}p < 0.05$, $^{***}p < 0.001$ versus control group.

Gin-Rb2 isolated from *P. ginseng* berry reduced the melanin content and tyrosinase and MITF protein levels in melan-a cells without cytotoxicity to the cells. Furthermore, it reduced the melanin synthesis and tyrosinase activity in the zebrafish as well.

Recently, various whitening studies have been conducted using natural products [11, 14], and predominant studies verified the depigmentation activity by inhibition of tyrosinase activation involved in melanin generation. To the best of our knowledge, the regulation of melanin synthesis is related to the action of the ERK (extracellular signal-regulated kinase) and PI3K (phosphatidylinositol 3-kinase)/Akt signaling pathways [29]. Activated ERK and PI3K/Akt signaling led to MITF phosphorylation and stimulated its degradation; thereby tyrosinase expression and melanogenesis were inhibited [22]. However, the role of Gin-Rb2 in melanogenesis has nat been clearly investigated yet. Consequently, further studies are needed to determine the exact molecular mechanisms of Gin-Rb2 action on the control of melanogenesis.

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