

## Inhibitory Effects of Neo Muscat Grape Vine Extracts on Melanin Biosynthesis

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**Neo Muscat grape vine extracts significantly inhibited melanin production in melanocyte stimulating hormone-stimulated B16 cells. They also exhibited tyrosinase inhibitory activity and reduced the levels of intra-cellular tyrosinase. Moreover, when the extracts were treated with UV B-induced hyper-pigmented brown guinea pig skin, depigmenting activity was observed, but with no visible edema. These results indicate that the vine extracts of Neo Muscat grapes might prove useful as a skin depigmenting agent.**

**Key words:** depigmenting agent, grape vine, melanin, tyrosinase

Grapes (*Vitis vinifera*) are the most important crop of the Vitaceae family, with the annual domestic grape production amounting to approximately 400,000 tons in Korea. Grapes are known to contain a number of polyphenol compounds, which exert various bio-regulatory effects [Cho *et al.*, 2003; Park and Oh, 2003; Yoo *et al.*, 2004; Heo *et al.*, 2007]. However, enormous quantity of the grape parts -including the skins, vines, and seeds- are mostly unused or simply utilized as fertilizer or animal feed. Thus, studies on the utilization of unused parts of grape are clearly warranted [Yoo *et al.*, 2004; Shin *et al.*, 2007]. Grape seeds have recently been shown to exert beneficial antioxidant effects, and several studies conducted to explore the uses of grape seeds, revealed that anti-inflammatory, anti-carcinogenic, and skin pigment inhibitory activities could be attributed to the grape seeds [Yamakoshi *et al.*, 2003; Terra *et al.*, 2007; Han *et al.*, 2008; Katiyar, 2008]. However, very few studies have been conducted thus far on the grape skin and vine.

Among the varieties of grape produced in Korea as of 2006, the Campbell Early variant accounts for approximately 70% of the total production, followed by the Kyoho,

Muscat Bailey A, and Neo Muscat varieties [Ministry of Agriculture and Forestry, 2007; Kim *et al.*, 2009]. Notably, the Neo Muscat grape (Green grape) is widely utilized as an ingredient of natural cosmetics, owing to its unique color.

As a component of the research currently being conducted on the unused parts of grapes, extracts of the skins, vines, and seeds of Neo Muscat were prepared. The inhibitory effects of these extracts on melanin biosynthesis were measured and compared using the melanocyte and brown guinea pig models. The results revealed that the grape vine extract exerts a marked inhibitory effect on melanin biosynthesis, and this novel finding is reported herein.

### Materials and Methods

**Grape samples and extraction.** Neo Muscat green grapes were harvested in late July of 2007 at Haman-gun, Gyeongsangnam-do. The collected samples were washed with distilled water, and the grape skins, vines, and seeds were separately crushed, followed by extraction for 1 h with 5 volumes of 80% ethanol per volume of sample at room temperature using an ultrasonic extractor (490W, Branson, Danbury, CT) in triplicate. The extracts were filtered through filter paper and concentrated with a rotary evaporator (Eyela, Tokyo, Japan) to prepare extracts of the Neo Muscat skins, vines, and seeds.

**Tyrosinase inhibitory activity.** Each concentration of the test substance was achieved by dissolving in MeOH.

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Subsequently, 120  $\mu$ L of L-dopa (5 mM, dissolved in 67 mM phosphate buffer, pH 6.8), and 40  $\mu$ L of either the same buffer or the test sample were added to 96-well microplates, followed by the addition of 40  $\mu$ L of mushroom tyrosinase (125 U). The amount of dopachrome in the reaction mixture was determined after 20 min incubation at 37°C. Based on the optical density at 490 nm (OD 490), the inhibitory activity of the sample was measured, with Kojic acid employed as the reference material.

**Cell culture and sample treatment.** B-16 cells were cultured in a DMEM culture medium containing 10% FBS and 1% antibiotics at 37°C and 5% CO<sub>2</sub>. When the cells achieved confluence, they were divided into 24-well plates at  $1 \times 10^5$  cells/well and stabilized for 24 h. They were then treated with  $\alpha$ -melanocyte-stimulating hormone (MSH) and the Neo Muscat samples.  $\alpha$ -MSH was added to a final concentration of 100 nM, and the Neo Muscat samples were prepared at concentrations of 10, 50, and 100  $\mu$ g/mL. The culture media were changed daily for 3 days and treated each time with  $\alpha$ -MSH and the Neo Muscat samples.

**Cell viability and melanin measurement.** Cell viability was measured using an MTT assay. For evaluation of the melanin production, the culture medium was drained and washed with PBS. The melanin was dissolved via the addition of 1 mL of 1 N NaOH. Absorbance was measured at 400 nm.

**Western immuno-blotting assay.** Cells were collected after centrifugation and washed with PBS. Lysis buffer (50 mM Tris-Cl pH 8.0, 10% SDS, 150 mM NaCl, 1% NP-40, 10  $\mu$ L/mL Apotinin) was added, maintained for 30 min at 4°C, and centrifuged at 13,000 rpm; the supernatant was used as a protein sample. Electrophoresis was conducted with 80  $\mu$ g protein on 10% polyacrylamide gel, blotted onto nitrocellulose membranes, and blocked with 5% skim milk. Tyrosinase primary antibodies (Santa Cruz Biotech) and anti-goat secondary antibodies (Santa Cruz Biotech) were added in turn and incubated. The bands were detected with an ECL kit (Amersham Pharmacia Biotech).

**Animals.** Brown guinea pigs weighing 450 g were purchased from SLC Inc. (Shizuoka, Japan), and individually housed in temperature- and moisture-controlled animal rooms under a 12 h light/dark cycle.

**UVB-induced hyperpigmentation in brown guinea pigs.** The guinea pigs were anesthetized with pentobarbital (30 mg/kg), and separate areas (1.5 cm diametrical circles) on the back of each animal were exposed to UV-B radiation (Waldmann UV 800, Herbert Waldmann GmbH, Philips TL/12 lamp emitting 280-305 nm). Total UV-B dosage was 500 mJ/cm<sup>2</sup> per exposure. Groups of 4 animals were used in the experiments. The animals were

**Table 1. Yields of Neo Muscat grape skin, vine, and seed extracts**

	Skin	Vine	Seed
Yields (%)	5.7	6.9	11.2

Each sample was extracted with 80% ethanol by sonication.

exposed to UV-B radiation once per week for 3 consecutive weeks. The Neo Muscat vine extract was then applied topically to the hyperpigmented areas (1% in EtOH/H<sub>2</sub>O = 7/3, 10  $\mu$ L/circle) once per day for 8 weeks from the day after the last tanning. The degree of pigmentation was evaluated by the L-values, which were measured with a chromameter (CR-300, Minolta).

**Statistical analysis.** The data are expressed as means  $\pm$  SD of at least three independent experiments.

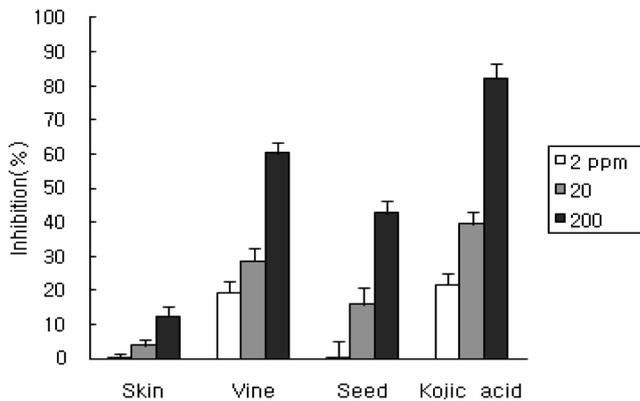
## Results

**Preparation of Neo Muscat extracts by parts.** The weights of the skins, vines, and seeds separated from a total of 10 kg of raw Neo Muscat grapes were 1.67, 0.24, and 0.23 kg, respectively. The yields of these extracts obtained by ultrasonic extraction using 80% ethanol and evaporation are shown in Table 1. The yield of the seed extract was highest at 11.2%, followed by that of the vine extract at 6.9%, and the skin extract showed the lowest yield.

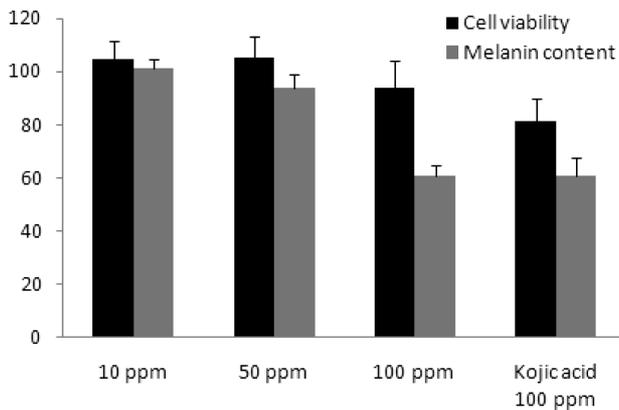
**Tyrosinase inhibitory activity.** The effects of the extracts of the Neo Muscat unused parts on tyrosinase activity, which is important at the early stages of melanin biosynthesis, are shown in Fig. 1. In particular, the vine extract evidenced the highest level of tyrosinase inhibitory activity of 60.3% at 200 ppm. The seed extract, which has previously been shown to exhibit tyrosinase inhibitory activity, though lower, also evidenced dose-dependent inhibitory effect of 46.2% at 200 ppm. No discernable tyrosinase inhibitory activity was found in the skin extract.

**Effects on cell viability and melanin production.** When vine extract was administered to mouse melanoma B16 cells for 3 days, results showed that the extract at 50 and 100 ppm did not induce significant cell death but rather reduced the melanin production by 11.6 and 33.2%, respectively (Fig. 2). These results indicated that decrease of melanin production by vine extract is not due to cell toxicity. Other parts of Neo Muscat, seed and skin, were reported to have severe cytotoxicity and no effect on melanin production, respectively [Lee, 2009].

**Effects of Neo Muscat samples on intracellular tyrosinase expression.** Western blotting was conducted after 3 days of treatment with the Neo Muscat samples in order to assess the degree of intracellular tyrosinase



**Fig. 1. Effects of Neo Muscat grape extract on tyrosinase inhibition.** Kojic acid was used as a positive control. Data are presented as the means of three distinct experiments  $\pm$ SD.

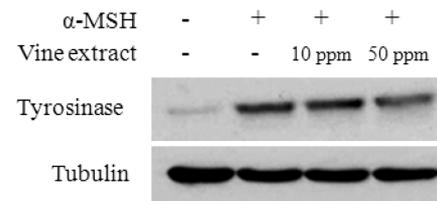


**Fig. 2. Effect of Neo Muscat grape vine extract on cell viability and melanin production in  $\alpha$ -MSH-stimulated B16 cells.** Data are presented as the means of three distinct experiments  $\pm$ SD.

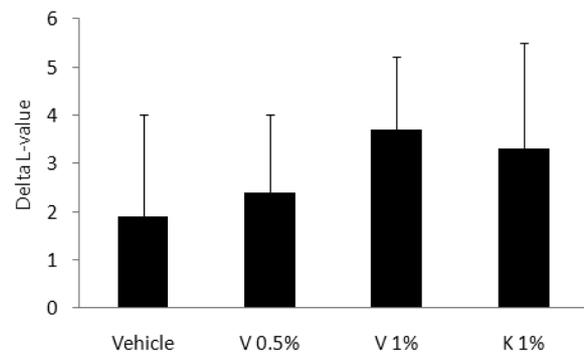
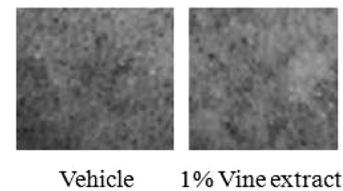
expression. The degree of tyrosinase expression was shown to increase after  $\alpha$ -MSH treatment, but tended to decrease after treatment with Neo Muscat vine extract at higher than 50 ppm (Fig. 3).

**Effects of Neo Muscat vine extract on Depigmenting activity of Brown Guinea Pig skin.** The changes in skin color resulting from an 8-week application of 1% Neo Muscat vine extract to brown guinea pig skin coupled with UV-B induced hyper-pigmentation, and the changes in the L value as measured by a chromameter (CR-200, Minolta) are shown in Fig. 4. The 1% Neo Muscat vine extract alleviated the UV-B induced hyperpigmentation of brown guinea pig skin, as compared to the vehicle, without showing any abnormal signs such as erythema or flare throughout the entirety of the experimental period.

### Discussion



**Fig. 3. Inhibitory effects of Neo Muscat grape vine extract on intracellular tyrosinase expression.** The changes in tyrosinase expression were measured after 3 days of treatment.



**Fig. 4. Depigmenting effects of Neo Muscat grape vine extract on UV-B induced hyper-pigmented brown guinea pig skin.** Delta L-values are expressed as the changes in the L-value after 8 weeks of application of Neo Muscat vine extracts. V, Vine extract; K, Kojic acid. Values are expressed as means  $\pm$ SD. N=5.

As part of an ongoing research effort into the utilization and properties of unused parts of grapes, several studies have been recently conducted concerning the inhibitory effects of grape seed extract on melanin production in the skin [Yamakoshi *et al.*, 2004; Lee, 2009; Zi *et al.*, 2009]. In this study, the unused parts of the Neo Muscat grape, which is utilized widely as an ingredient in cosmetics due to its characteristic color, were investigated in order to evaluate their inhibitory effects on melanin production. The results confirmed that the vine extract exerted a profound inhibitory effect on melanin production. Cho *et al.* [2003] reported that vine has a higher amount of resveratrol than the other parts.

In our results, the Neo Muscat vine extract attenuated melanin production of the melanoma B16 cells in a dose-

dependent manner, without any discernable cytotoxicity. This inhibitory effect on melanin production may be attributable to the inhibitory effects of tyrosinase activity and the intracellular expression; therefore, additional studies on tyrosinase-related proteins 1 and 2, and microphthalmia-associated transcription factor will be conducted in the future. Furthermore, the Neo Muscat vine extract alleviated the skin color of hyperpigmented brown guinea pigs without inducing any abnormal responses such as erythema or flare. According to the aforementioned results, the Neo Muscat vine extract can be considered extremely useful as a low-cytotoxic, skin pigmentation-inhibitory material.

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