Melanogenesis-Promoting Effects of *Rhynchosia nulubilis* and *Rhynchosia volubilis* Ethanol Extracts in Melan-a Cells

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We evaluated the antioxidant activity and melanogenic effects of black soybean ethanol extracts, including *Rhynchosia nulubilis* bean ethanol extract (RNBEE), *R. nulubilis* leaf ethanol extract (RNLEE), *R. volubilis* bean ethanol extract (RVBEE), and *R. volubilis* leaf ethanol extract (RVLEE). The total polyphenol contents of RNBEE, RNLEE, RVBEE, and RVLEE were 16.0, 57.7, 365.9, and 260.1 mg/g, respectively. The total flavonoid contents of RNBEE, RNLEE, RVBEE, and RVLEE were 40.4, 91.7, 84.7, and 216.5 mg/g, respectively. The electron-donating abilities of RNBEE, RNLEE, RVBEE, and RVLEE at 1,000 µg/mL were 32.4%, 12.7%, 83.5%, and 84.5%, respectively. RNBEE, RNLEE, RVBEE, and RVLEE at 50 µg/mL significantly increased ($p < 0.01$) melanin contents by 30.4%, 32.1%, 35.5%, and 37.4%, respectively, compared to that of the control. RNBEE, RNLEE, RVBEE, and RVLEE at 50 µg/mL significantly increased ($p < 0.01$) intracellular tyrosinase activity by 18.4%, 21.8%, 21.5%, and 21.1%, respectively, compared to that of the control. These results demonstrated that black soybean ethanol extracts promote melanogenesis in melan-a cells. Among the black soybean ethanol extracts, *R. volubilis* was found to be more effective than *R. nulubilis*, and leaf extract was found to be more effective than bean extract. The potential mechanism underlying the hyperpigmentation effects of black soybeans is the promotion of tyrosinase activity.

Key words: Antioxidant activity, Black soybean ethanol extracts, Melan-a cells, Melanogenesis, Tyrosinase activity

INTRODUCTION

Graying of hair or canities occurs in all individuals regardless of sex or race and has a positive correlation with chronological aging (1). Recently, premature canities has been increasingly observed in young individuals. Canities is a consequence of decreased melanin synthesis, which leads to the impairment of hair follicle function, but can also be triggered by numerous other intrinsic factors including general metabolism and nutritional status, hair-cycle-dependent changes, variable hormone-responsiveness, genetic defects,

and age-associated changes (2).

Melanocytes produce the pigment melanin that governs hair color. Within the basal portion of hair follicles, the hair matrix, composed of keratinocytes and melanocytes, grows downward during anagen (3). Analysis of melanocytes during the murine hair cycle revealed that melanocytes could be lost from the hair bulb via apoptosis during hair follicle involution or catagen (4). Ultimately, this can lead to exhaustion of the melanocyte stem cell pool in the bulge region of the hair follicle (5). Two types of melanin are produced by melanocytes: yellow-red pheomelanins and brown-black eumelanins (6). However, there has been no conclusive proof thus far that any of the above-mentioned melanocyte functions are relevant to common graying. Therefore, whether or not apoptosis occurs in aging hair follicle melanocytes and its underlying causes remain unclear (7).

Melanin synthesis is mainly regulated by the tyrosinase. Hair graying results from reduction in tyrosinase activity of hair bulbar melanocytes due to the cytotoxic oxidative nature of melanin biosynthesis, suboptimal melanocyte cortical

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keratinocyte interactions, and defective migration of melanocytes from a reservoir in the upper outer root sheath to the pigment-permitting microenvironment close to the dermal papilla of the hair bulb (1,8).

For more than 100 years, paraphenylenediamine (PPD) and other related members of the aromatic amine family have been the main agents used in permanent hair dyes accounting for more than two-thirds of the commercially manufactured hair dyes (9). PPD causes contact dermatitis in susceptible individuals, but when ingested, causes acute angioedema of face and neck, rhabdomyolysis, and acute renal failure (10,11). Therefore, there is a continued need for alternative methods that can inhibit hair graying using materials that increase tyrosinase activity and melanin synthesis with a less toxic effect on melanocytes.

Soybean (*Glycine maxim* L. Merrill) seed coats have various colors including yellow, red, green, and black (12). The SoRiTae (*G. maxim*) and Yakong (*Rhynchosia nulubilis* and *R. volubilis*) are examples of black soybean and black soybean can be further classified into those with yellow or green cotyledon according to cotyledon color (13). SoRiTae (*G. maxim*) has a yellow cotyledon and Yakong (*R. nulubilis* and *R. volubilis*) has a green cotyledon.

R. volubilis, a less famous species, has smaller bean than *R. nulubilis*. Yoshida *et al.* (14) reported that soybean cultivars with black seed coats contain anthocyanins, cyanidin-3-glucoside being the dominant component. In addition, black soybeans are superior inhibitors of low-density lipoprotein oxidation compared to yellow soybeans (15). The ferric reducing antioxidant power, free radical-scavenging effect, and total phenolic contents of black soybeans are comparatively higher than those in yellow soybeans (16-18). Lee *et al.* (19) reported that yellow soybeans (*G. maxim*) markedly increased melanogenesis by increasing tyrosinase activity. However, the ability of black soybeans (*G. maxim*) to inhibit tyrosinase activity was also reported (20).

This study was conducted to evaluate the antioxidant activity of *R. nulubilis* ethanol extract (RNEE) and *R. volubilis* ethanol extract (RVEE) and their melanogenic effects on melanin synthesis and tyrosinase activity in melan-a cells.

MATERIALS AND METHODS

Reagents and apparatus. Dimethyl sulfoxide (DMSO), 2,6-di-tert-butylate hydroxytoluene (BHT), 3,4-dihydroxy-L-phenyl-alanine (L-DOPA), 1,1-diphenyl-2-picryl hydrazyl (DPPH), 3-isobutyl-1-methylxanthine (IBMX), tannic acid, L-tyrosine, Folin-Ciocalteu's phenol reagent, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) and 12-*o*-tetradecanoyl-phorbol-13-acetate (TPA) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), Roswell Park Memorial Institute (RPMI)-1640 medium, fetal bovine serum (FBS)

and penicillin/streptomycin mixture (P/S) were obtained from Lonza (Cascade, MD, USA). Inverted microscope (CKX41, Olympus, Tokyo, Japan) was used to observe cell growth and CO₂ incubator (MCO-17AC, Sanyo electric, Osaka, Japan) was used for cell culture.

Preparation of RNEE and RVEE. The beans and leaves of *R. nulubilis* were collected from Yeacheon, Kyungbook and those of *R. volubilis* were collected from Gotjawal, Jeju Iland. Pulverized samples (50 g) were put into a flask and extracted thrice with 500 mL of 80% ethanol for 24 hr each at 25°C. The extract was filtered through filter paper and concentrated using rotary vacuum evaporator followed by lyophilization. The yield of the leaf and bean extracts of *R. nulubilis* were 16.8% and 10.1%, respectively, while those of *R. volubilis* were 9.8% and 11.0%, respectively.

Total polyphenol content. Total polyphenol contents of RNEE and RVEE were measured using the Folin and Denis assay (21). RNEE and RVEE were dissolved in 1 mL distilled water and placed in test tubes. Folin-reagent (1 mL) was added and the tubes were allowed to stand for 3 min. Following addition of 1 mL of 10% Na₂CO₃, the mixture was shaken vigorously and allowed to stand for 60 min. The absorbance was measured at 725 nm and the polyphenol content was quantified using a standard curve prepared using tannic acid.

Total flavonoid content. Total flavonoid contents of RNEE and RVEE were measured using method of Davies *et al.* (22) with modification. Samples of extracts (100 µL each of RNEE or RVEE) were mixed with 1 mL of di-ethylene glycol reagent and 100 µL of 1 N NaOH in test tubes. The mixture was shaken vigorously and reacted at 37°C for 60 min before measuring the absorbance at 420 nm. The standard curve was prepared using rutin.

Electron-donating ability. DPPH radical scavenging effect was evaluated according to the method of Blois (23). RNEE and RVEE were dissolved in distilled water to a range of final concentrations (100, 500, and 1000 µg/mL) each. Test agent solution (1 mL) was placed in each test tube, followed by the addition of 4 mL of 4 × 10⁻⁴ M DPPH. The mixture was shaken vigorously and then left for 10 s at 60°C before measuring the absorbance at 525 nm. BHT was used as the positive control.

Cell culture. The melan-a cells (passages 30-37) used in this study were obtained from Dr. Dorothy Bennett (St. George's Hospital, UK). These highly pigmented and immortalized cells were derived from C57BL/6 mice. Cells were grown in RPMI-1640 medium, supplemented with 10% FBS, 1% P/S, and 200 nM TPA at 37°C and 10% CO₂ in an incubator for 72 hr.

MTT assay. Cell viability was assessed by the MTT assay. The melan-a cells were seeded in a 96-well plate (0.5×10^4 cells/well) and grown in the incubator at 37°C and 10% CO₂ for 24 hr. Aliquots (200 µL) of RNEE and RVEE, diluted with RPMI-1640 medium to a range of concentrations (25, 50, 100, and 200 µg/mL), were added to the wells and the cells were grown in the incubator at 37°C and 10% CO₂ for 48 hr. Cells were then placed in a medium containing 0.5 µg/mL MTT and grown in the incubator at 37°C and 10% CO₂ for 3 hr. Following centrifugation of the plate at $200 \times g$ for 10 min, the cells settled and the medium was removed. DMSO (200 µL) was added to each well and the cells were resuspended for 15 min in a plate-shaker. Absorbance was measured at 540 nm using a plate reader (680, Bio-Rad, Tokyo, Japan).

Morphological observation of melan-a cells. Melan-a cells were treated with increasing concentrations of RNEE or RVEE (10, 50, 100, and 200 µg/mL) to observe any effect on their morphology, and grown in the incubator at 37°C and 10% CO₂ for 48 hr. Following incubation, the medium was changed and the cells were observed under an inverted microscope.

Melanin assay. The melan-a cells were distributed in a 48-well plate (2×10^4 cells/well) and grown in the incubator at 37°C and 10% CO₂ for 24 hr. Following incubation, 500 µL of RNEE and RVEE diluted with RPMI-1640 medium to a range of concentrations (25, 50, 100, and 200 µg/mL) were added to the wells and the cells were grown in the incubator at 37°C and 10% CO₂ for 72 hr, and then washed. The same treatment was repeated once more. Next, melanin was dissolved in 1 N NaOH, and the absorbance was measured at 490 nm using a plate reader.

Intracellular tyrosinase activity assay. The melan-a cells were distributed in a 60-mm cell culture dish (4×10^5 cells/well), and grown in the incubator at 37°C and 10% CO₂ for 24 hr. RNEE and RVEE (5 mL) were diluted with RPMI-1640 medium to a range of concentrations (25, 50, 100, and 200 µg/mL) and added to the dish. Cells were grown in the incubator at 37°C and 10% CO₂ for 48 hr. The cells were washed with phosphate-buffered saline (PBS), detached with 200 µL of 1% Triton X-100, transferred to Eppendorf tubes, subjected to extraction on ice with agitation six times every 10 min, and then centrifuged at 4°C at $18,000 \times g$ for 20 min. Following centrifugation, 100 µL of 0.2% L-DOPA was added and the mixture was placed in the incubator at 37°C and 10% CO₂ for 1 hr, and absorbance was measured at 490 nm using a plate reader.

Statistical analysis. Statistical analysis was performed using SPSS 21.0 for Windows (IBM, Armonk, NY, USA). The difference between two groups was analyzed by Stu-

dent's *t*-test. *P* values < 0.05 were considered to be statistically significant.

RESULTS

Antioxidant activity of the black soybean extracts.

The total polyphenol contents of RNBEE, RNLEE, RVBEE, and RVLEE were 16.0, 57.7, 365.9, and 260.1 mg/g, respectively, according to the standard curve obtained using tannic acid (Fig. 1). The total flavonoid contents of RNBEE, RNLEE, RVBEE, and RVLEE were 40.4, 91.7, 84.7, and 216.5 mg/g, respectively, according to the standard curve constructed linear using rutin (Fig. 1). Electron-donating

Fig. 1. Total polyphenol and flavonoid contents of RNBEE, RNLEE, RVBEE, and RVLEE. RNBEE: *Rhynchosia nulubilis* bean ethanol extract, RNLEE: *Rhynchosia nulubilis* leaf ethanol extract, RVBEE: *Rhynchosia volubilis* bean ethanol extract, RVLEE: *Rhynchosia volubilis* leaf ethanol extract. Values are the mean $\pm$ SD of three independent measurements.

Fig. 2. Electron-donating ability of RNBEE, RNLEE, RVBEE, and RVLEE. Each substance was evaluated for its ability to donate electrons to the free radical DPPH. AA: Ascorbic acid, RNBEE: *Rhynchosia nulubilis* bean ethanol extract, RNLEE: *Rhynchosia nulubilis* leaf ethanol extract, RVBEE: *Rhynchosia volubilis* bean ethanol extract, RVLEE: *Rhynchosia volubilis* leaf ethanol extract. Values are the mean $\pm$ SD of three independent measurements.

Fig. 3. Effect of RNBEE, RNLEE, RVBEE, and RVLEE on viability of melan-a cells. Viability of melan-a cells treated with test materials (0~200 µg/mL) for 48 hr was analyzed by MTT assay. V: vehicle, RNBEE: *Rhynchosia nulubilis* bean ethanol extract, RNLEE: *Rhynchosia nulubilis* leaf ethanol extract, RVBEE: *Rhynchosia volubilis* bean ethanol extract, RVLEE: *Rhynchosia volubilis* leaf ethanol extract. Values are the mean $\pm$ SD of three independent measurements.

abilities of RNBEE, RNLEE, RVBEE, and RVLEE at 1,000 µg/mL were 32.4%, 12.7%, 83.5%, and 84.5%, respectively (Fig. 2). These values were lower than that of ascorbic acid (94.5%) at the same concentration.

Effects of the black soybean extracts on melan-a cell viability.

The MTT assay was used to determine the maximum permissible level (MPL) of RNEE and RVEE in melan-a cells. RNBEE showed the viability of melan-a cells above 80% at concentrations between 6.25 and 200 µg/mL, compared to the vehicle (Fig. 3). RNLEE showed the cell viability above 80% at concentrations between 6.25 and 100 µg/mL; however, it decreased the cell viability by 36.6% at 200 µg/mL. RVBEE and RVLEE at the concentration of 50 µg/mL showed the viability above 80%. However, cell viability at 100 µg/mL reduced by 47.4% and 35.7%, respectively. The MPL for RNBEE, RNLEE, RVBEE, and RVLEE in melan-a cells was 200, 100, 50, and 50 µg/mL, respectively. IBMX showed the cell viability above 80% at concentrations of 6.25 and 12.5 µg/mL; however, it decreased the cell viability by 25.8% at 25 µg/mL, showing the MPL

Fig. 4. Morphological changes of melan-a cells treated with RNBEE, RNLEE, RVBEE, and RVLEE. Morphological changes of melan-a cells treated with 12.5 µg/mL IBMX (B), 50 µg/mL RNBEE (C), 50 µg/mL RNLEE (D), 50 µg/mL RVBEE (E), and 50 µg/mL RVLEE (F) for 48 hr were compared to those of untreated control (A). 200 $\times$ magnification.

Fig. 5. Melanogenic effect of RNBEE, RNLEE, RVBEE, and RVLEE on melan-a cells. Melanin production in the cells treated with test materials (6.25~50 µg/mL) for 72 hr was measured by ELISA. C: control, PC: 12.5 µg/mL IBMX, RNBEE: *Rhynchosia nulubilis* bean ethanol extract, RNLEE: *Rhynchosia nulubilis* leaf ethanol extract, RVBEE: *Rhynchosia volubilis* bean ethanol extract, RVLEE: *Rhynchosia volubilis* leaf ethanol extract. Values are the mean $\pm$ SD of three independent measurements. The values with an asterisk are significantly different from the control group by Student's *t*-test (* p < 0.05, ** p < 0.01).

of 12.5 µg/mL (data not shown). IBMX (12.5 µg/mL), and RNBEE, RNLEE, RVBEE, and RVLEE at the concentration of 50 µg/mL resulted in increased accumulation of melanin and melanocyte dendrites in melan-a cells compared to those observed in untreated control (Fig. 4).

Effects of the black soybean extracts on melanin synthesis. In comparison with the control group, the melanin contents of RNBEE, RNLEE, RVBEE, and RVLEE-treated groups significantly increased (p < 0.01) at 50 µg/mL by 30.4%, 32.1%, 35.5%, and 37.4%, respectively (Fig. 5). IBMX (PC) at 12.5 µg/mL also significantly (p < 0.01) increased the melanin contents by 51.3%, compared to the control.

Effects of the black soybean extracts on tyrosinase activity. In comparison with the control treatment, treatment with RNBEE, RNLEE, RVBEE, and RVLEE at 50 µg/mL significantly increased (p < 0.01) intracellular tyrosinase activities by 18.4%, 21.8%, 21.5%, and 21.1%, respectively (Fig. 6). IBMX at 12.5 µg/mL also significantly increased the tyrosinase activity by 67.4% (p < 0.001).

DISCUSSION

A form of transient “canities” occurs at the end of every hair growth cycle. Small numbers of dendritic melanocytes can be detected in the retreating epithelial strand of catagen undergoing active resorption via apoptosis (2). “Free radi-

Fig. 6. Effect of RNBEE, RNLEE, RVBEE, and RVLEE on intracellular tyrosinase activity in melan-a cells. Intracellular tyrosinase activity in the cells treated with test materials (6.25~50 µg/mL) for 48 hr was measured by ELISA. C: control, PC: 12.5 µg/mL IBMX, RNBEE: *Rhynchosia nulubilis* bean ethanol extract, RNLEE: *Rhynchosia nulubilis* leaf ethanol extract, RVBEE: *Rhynchosia volubilis* bean ethanol extract, RVLEE: *Rhynchosia volubilis* leaf ethanol extract. Values are the mean $\pm$ SD of three independent measurements. The values with an asterisk are significantly different from the control group by Student's *t*-test (* p < 0.05, ** p < 0.01, *** p < 0.001).

cal” theory of aging is by far the most popular theory for skin aging and can also be easily verified *in vitro*. Based on the theory, the extraordinary melanogenic activity of a limited number of pigmented bulbar melanocytes, in some hair follicles, can most likely generate large amounts of reactive oxygen species (ROS) via the oxidation of tyrosine and DOPA to melanin. It is most likely that the remarkably high levels of follicular melanogenesis would need to be regulated by an efficient antioxidant system. If not adequately removed, accumulation of these ROS would induce nuclear and mitochondrial DNA damages, ultimately resulting in the accumulation of mutations and induction of oxidative stress (8). Therefore, more studies on antioxidant and electron transfer systems are needed for further progress in this field.

Total polyphenol and flavonoid contents have been widely used either to assess the free radical-scavenging activity or to study the antioxidant activity of plant extracts. Total polyphenol and flavonoid contents and electron-donating ability of RNBEE, RNLEE, RVBEE, and RVLEE were determined to assess the anti-oxidative capacity. The total polyphenol contents of RNBEE, RNLEE, RVBEE, and RVLEE were 16.0, 57.7, 365.9, and 260.1 mg/g, respectively; the total flavonoid contents of those were 40.4, 91.7, 84.7, and 216.5 mg/g, respectively, RVEE had higher total polyphenol and flavonoid contents than RNEE. According to Hong *et al.* (24), the total polyphenol and flavonoid contents of black soybean (*R. nulubilis*) water extract were 23.0

and 30.5 mg/g, respectively. Therefore, RNEE and RVEE have a higher antioxidant activity than *R. nulubilis* water extract. In the current study, electron-donating abilities of RNBEE, RNLEE, RVBEE, and RVLEE at 1,000 µg/mL were 32.4%, 12.7%, 83.5%, and 84.5%, respectively. According to Ralison (25), DPPH radical-scavenging ability of black soybean (*G. maxim*) at 1,000 µg/mL was 25%. RNBEE, RVBEE and RVLEE have a higher radical-scavenging capacity than black soybean (*G. maxim*). These results indicate that RNBEE, RNLEE, RVBEE, and RVLEE are valuable natural antioxidants.

Melan-a cells are an immortal cell line of pigmented melanocytes commonly used in the study of melanin biosynthesis (26). In the present study, the effect of RNEE and RVEE on hair graying was evaluated using melan-a cells derived from C57BL/6 mice. Lee et al. (19) reported that melanin contents in melan-a cells significantly increased by 51.2% after treatment with 12.5 µg/mL of yellow soybean (*G. maxim*) methanol extract. In the current study, RNBEE, RNLEE, RVBEE, and RVLEE-treated groups showed a dose-dependent increase in melanin levels, although the increase observed was not as prominent as that observed by Lee et al. (19). In addition, RVEE had greater melanogenic effects than RNEE.

Tyrosinase is the rate-limiting enzyme for melanin biosynthesis; therefore, the effect of RNEE and RVEE on intracellular tyrosinase activity was determined. According to Lee et al. (19), yellow soybean (*G. maxim*) methanol extract increased the intracellular tyrosinase activity in a dose-dependent manner. In contrast, Lai et al. (27) reported that black soybean (*G. maxim*) water extract from sprouts grown to 0.5 cm inhibited the mushroom tyrosinase activity by 40% at 2,000 µg/mL. In the current study, in comparison with the control group, RNBEE, RNLEE, RVBEE, and RVLEE at 50 µg/mL significantly increased intracellular tyrosinase activity ($p < 0.01$).

In conclusion, we verified that RNBEE, RNLEE, RVBEE, and RVLEE promote melanin synthesis by increasing tyrosinase activity. *R. volubilis* is more effective than *R. nulubilis* and the leaf is more effective than the bean. Among these, RVLEE may be the most effective hyperpigmentation agent with lower cytotoxicity than IBMX.

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