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Down-Regulation of Tyrosinase, TRP-2 and MITF Expressions by *Neolitsea aciculata* Extract in Murine B16 F10 Melanoma

¹S.S. Kim, ²W.J. Yoon, ²C.G. Hyun and ¹N.H. Lee

¹Department of Chemistry, Cheju National University, Jeju 690-756, Republic of Korea

²Jeju Biodiversity Research Institute and Jeju Hi-Tech Industry Development Institute,
Jeju 699-943, Republic of Korea

Abstract: Melanogenesis is a well known physiological response of human skin and is mainly caused by ultraviolet irradiation and some genetic factors. In our efforts to find new skin lightening agents, *Neolitsea aciculata* butanol fraction (NAB) was investigated for its ability to inhibit melanogenesis. In this study, NAB's effect on inhibition of mushroom tyrosinase was assessed, which indicated its good anti-tyrosinase activity (IC_{50} 211.4 Fg mL⁻¹). Furthermore, NAB was shown to down-regulate melanin content on a dose dependent pattern. To clarify the target of its action in melanogenesis, Western blotting for tyrosinase, tyrosinase-related protein-1 (TRP-1), TRP-2 and microphthalmia-associated transcription factor (MITF) was performed. As a result, NAB inhibited tyrosinase, TRP-2 and MITF expressions in a dose-dependent manner. However, it did not inhibit TRP-1 expression. In order to evaluate its potential in cosmetic applications, NAB's cell toxicity was also determined by MTT assays using human dermal fibroblast cells. The NAB exhibited low cytotoxicity at 100 µg mL⁻¹ in human cell line. Therefore, this study suggests that NAB is a potential anti-melanogenic agent and might be effective in treatment of hyperpigmentation disorders.

Key words: Melanogenesis, *Neolitsea aciculata*, tyrosinase, TRP-1, TRP-2, MITF

INTRODUCTION

Mammals skin pigmentation results from the melanin production in melanocytes and its accumulation in the epidermis. Melanin synthesis or melanogenesis is a complex pathway involving enzymatic and chemical reactions, which occurs in melanosomes containing all components required to synthesize pigment (Slominski *et al.*, 2004; Okombi *et al.*, 2006). Three melanocyte-specific enzymes, tyrosinase, tyrosinase-related protein-1 (TRP-1) and TRP-2 are involved in melanogenesis, in which L-tyrosine is converted into melanin polymer (Laskin and Piccinin, 1986; Busca and Ballotti, 2000). Tyrosinase enzyme, in particular, catalyzes the oxidations in two rate-limiting steps in this process i.e., the hydroxylation of L-tyrosine to 3,4-dihydroxyphenylalanine (DOPA) and the subsequent oxidation of DOPA to dopaquinone. Therefore, tyrosinase inhibitors find applications in cosmetic products for whitening after sunburn as well as in the treatment of dermatological disorders related to melanin hyperpigmentation (Kazuhisa and Minoru, 1991; Karioti *et al.*, 2007). Even though natural and synthetic compounds exhibiting anti-tyrosinase activities were

continuously identified, relatively few of them are used as skin-whitening agents mainly due to safety concerns. For example, kojic acid was among the most popular before serious side effects came to limit their human use (Okombi *et al.*, 2006). With this regard, development of safe and effective novel whitening agents is still necessary. *Neolitsea aciculata* (Bl.) Koidz is an evergreen shrub in the laurel family Lauraceae and is mainly growing in Southern part of Korea including Jeju Island. This trees were widely used as raw materials for building the ship and making the charcoals. Their phytochemical analysis as well as biological efficacy regarding depigmenting effects have not yet been examined. We are continuously investigating the plants in Jeju Island to develop biologically active natural products (Ko *et al.*, 2009a, b; Sultan and Lee, 2009). In this study, we focused on evaluating the inhibitory effects of *N. aciculata* on melanin biosynthesis and its potential application for natural cosmetic ingredients.

MATERIALS AND METHODS

Plant material: The stems of *N. aciculata* were prepared on Jeju Island from May 2008 and then identified by

Dr. Dae-Sin Kim. A voucher specimen (No. 068) is deposited at the Natural Product Chemistry Laboratory, Department of Chemistry, Cheju National University, Korea.

Preparation of *Neolitsea aciculata* butanol fraction

(NAB): The stems of *N. aciculata* were washed, dried in the shade and ground by a mill into a fine powder. The powder (1 kg) was extracted with 80% ethanol by stirring with a magnetic stirrer at room temperature for 24 h. The extracts was filtered to remove insoluble residue and the filtrate was concentrated by evaporator under reduced pressure at 40°C. The concentrated ethanol extract (50 g) was suspended in water (2 L) and partitioned successively with n-hexane (2 L), ethyl acetate (EtOAc, 2 L) and n-butanol (BuOH, 2 L). This partition procedures were repeated three times to yield the butanol fraction (NAB, 9.4 g, 18.9%), along with n-hexane (2 g, 4.0%), EtOAc (8 g, 16.0%) and H₂O (16.8 g, 33.6%) fractions.

Cell culture: Mouse melanocyte B16F10 was purchased from the Korean Cell Line Bank (KCLB, Seoul, Korea). Human dermal fibroblast cells were acquired from the Amore-Pacific Corporation R and D Center, Korea. B16F10 murine melanoma cells and human dermal fibroblast cells were cultured in Dulbecco's modified Eagles medium (DMEM) supplemented with 10% fetal bovine serum (GIBCO, Inc., NY, USA) and 1% penicillin-streptomycin at 37°C in a humidified 95% air and 5% CO₂ atmosphere.

Cell viability determination: Cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were seeded on 96-well plates and drug treatment began 24 h after seeding (Mosmann, 1983; Page *et al.*, 1993). The B16F10 murine melanoma cells and human dermal fibroblast cells were incubated with various concentration of NAB for 48 h at 37°C in a humidified 95% air and 5% CO₂ atmosphere. The MTT (1 mg mL⁻¹ in phosphate-buffer saline, PBS) was added to each well in a 1/10 volume of medium. Cells were incubated at 37°C for 4 h. Finally, the supernatant was removed and the formazan crystals were dissolved in DMSO. Absorbance was measured at 570 nm. Percent of cells showing cytotoxicity was determined relative to the control group.

Measurement of melanin content: Melanin content was measured as reported by Friedmann and Gilchrest with slight modifications (Friedmann and Gilchrest, 1987). The cells were treated with NAB (12.5, 25, 50 or 100 Fg mL⁻¹) and α -MSH (50 nM) for 3 days. After treatment, the cells were detached by incubation in

trypsin/ethylenediaminetetraacetic acid. After precipitation, the color of the cell pellets were evaluated visually and cell pellets containing a known number of cells were solubilized in boiling 1 M NaOH for 1 h. Spectrophotometric analysis of melanin content was performed at 475 nm absorbance.

Assay to measure inhibitory effects on mushroom

tyrosinase: Mushroom-derived tyrosinase was used as the source of the enzyme for the entire study. Tyrosinase activity was determined as described previously with minor modification (Friedmann *et al.*, 1990). Briefly, each sample was assayed for tyrosinase inhibition by measuring its effect on the tyrosinase activity using a 96-well reader (Bio-Tek Instruments, Inc, Winooski, VT). The test reaction was carried out in 100 mM sodium phosphate buffer (pH 6.8) containing 0.2 mM L-tyrosine and 80 unit mL⁻¹ mushroom tyrosinase at 37°C. The reaction mixture was pre-incubated for 10 min before adding the substrate. The change in the absorbance at 475 nm was measured. The same mixture, except for the plant extract, was used as the control. Arbutin and kojic acid were used as positive controls. Each treatment was replicated thrice. The percent inhibition of tyrosinase activity was calculated as follows:

$$\text{Inhibition (\%)} = \frac{1 - (\text{Abs}_{\text{sample}} - \text{Abs}_{\text{blank}})}{\text{Abs}_{\text{control}}} \times 100$$

where, Abs_{sample} is the absorbance of the experimental sample, Abs_{blank} is the absorbance of the blank, Abs_{control} is the absorbance of the control. The concentration of a compound at which 50% of the enzyme activity was inhibited (IC₅₀ value) was obtained by linear curve fitting.

Measurement of tyrosinase, TRP-1, TRP-2 and MITF in melanoma B16F10 cells by Western blot:

To determine the amount of tyrosinase and tyrosinase-related protein expression, Western blotting analysis was performed (Park *et al.*, 2004). The B16F10 melanoma cells stimulated by α -MSH (50 nM) were treated with NAB (12.5, 25, 50 or 100 Fg mL⁻¹) for 3 days. After treatment, the cells were collected and lysed with cell lysis buffer [50 mM TrisBHCl (pH 6.8), 2% SDS, 6% mercaptoethanol, 1% glycerol]. Whole cell lysates (5×10⁴ cells equivalents per lane) were separated by 7.5% SDS-polyacrylamide gel electrophoresis as described previously and transferred to a PVDF membrane. The membrane was blocked with 5% skimmed milk in phosphate-buffered saline containing 0.05% Tween 20. Tyrosinase, TRP-1, TRP-2 and MITF bands were detected respectively with the rabbit polyclonal anti-tyrosinase antibody (dilution 1:1000),

anti-TRP-1 antibody (dilution 1:1000), anti-TRP-2 antibody and anti-MITF antibody (dilution 1:500) purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and then further incubated with horseradish peroxidase-conjugated anti-rabbit IgG antibody at a 1:5000 dilution. Bound antibodies were detected using an enhanced chemiluminescence kit (Amersham). Loading control was assessed using anti- β -actin antibody. Positive bands were analyzed using a gel image analysis instrument.

Statistical analysis: All data were obtained in triplicate and are represented as Means \pm SE. Significant differences between treatments were determined by the Student's t-test in one-way Analysis of Variance (ANOVA).

RESULTS AND DISCUSSION

Tyrosinase inhibition, cell viability and melanin content after treatment with NAB: Mushroom tyrosinase has been widely used as the target enzyme for screening and characterizing potential tyrosinase inhibitors. The monophenolase activity of this enzyme was assayed using L-tyrosine as substrate (Espin *et al.*, 2006). The progress curve of enzyme reaction was a line passing through the origin without lag period. The formation of product was in proportion to the reaction time. The monophenolase activity was estimated by the value of the line slope in the graph. During the preliminary screening with mushroom tyrosinase, we observed that the 80% ethanol extract of *N. aciculata* stem showed a significant inhibitory effect on L-tyrosine oxidation. The ethanol extract was partitioned sequentially into n-hexane, ethyl acetate, n-butanol and water. As each solvent fraction was subjected to tyrosinase enzyme screening, n-butanol fraction (NAB) showed particularly strong inhibitory activity. Little or no activity was observed from the n-hexane, ethyl acetate and water fractions (data not shown). As shown in Fig. 1, the monophenolase activity of mushroom tyrosinase decreased in a dose-dependent manner with increasing the NAB concentrations. The inhibitory concentrations (IC_{50}) leading to 50% activity lost were determined as 211.4 Fg mL⁻¹ (Fig. 1).

In the present study, B16F10 murine melanoma cell was also used for examining the NAB's inhibitory effect on melanogenesis. To examine test samples' cytotoxic effects on this melanoma cell, an MTT assay was conducted first. These results showed that the cell viability rates reached to almost 100 and 80%, respectively by addition of 50 and 100 Fg mL⁻¹ NAB on B16F10 melanoma cells, which suggests that NAB did not affect cell viability after treatment for 72 h. Thus, given the low cytotoxic effect of NAB on B16 melanoma cell, the inhibitory effects of NAB on melanin production were

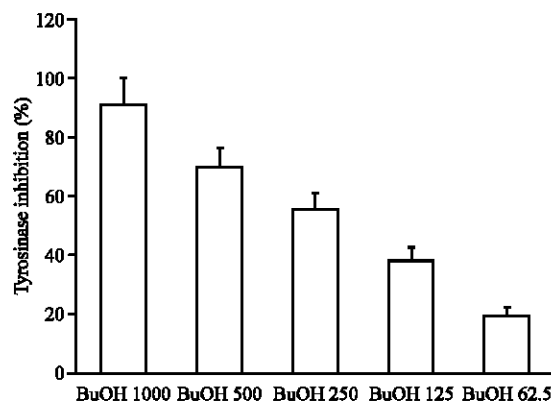


Fig. 1: Effects of NAB on mushroom tyrosinase activity. Data are expressed as a percentage of control and are Mean \pm SEM of triplicated experiments

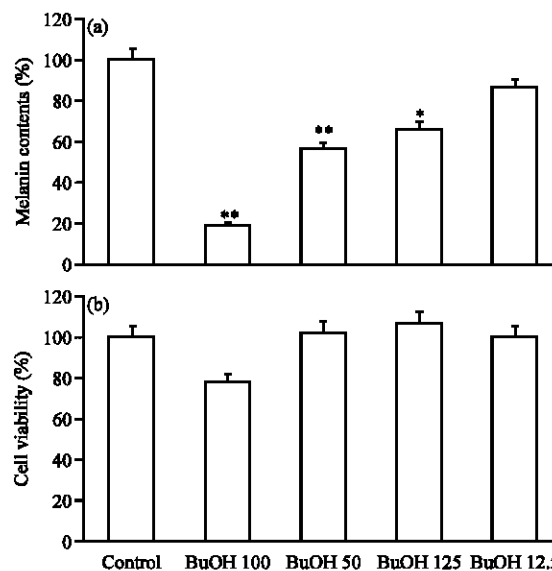


Fig. 2: (a, b) Effects of NAB on melanin contents and cytotoxicity. After incubation of B16F10 murine melanoma cells with various concentrations of NAB for 72 h, melanin contents and cytotoxicities are determined. Data are expressed as a percentage of control and are Mean \pm SEM of triplicated experiments. *p = 0.05, **p = 0.01

assessed. The melanin contents produced in melanocytes for NAB-treated samples are shown in Fig. 2a and b. Compared to the control group, treatment with NAB (12.5, 25, 50, 100 Fg mL⁻¹) for 72 h reduced melanin productions on a dose dependent pattern.

Effects of NAB on tyrosinase, TRP-1, TRP-2 and MITF protein expression in murine B16F10 melanoma cell: In order to further understand the hyperpigmentary effect of

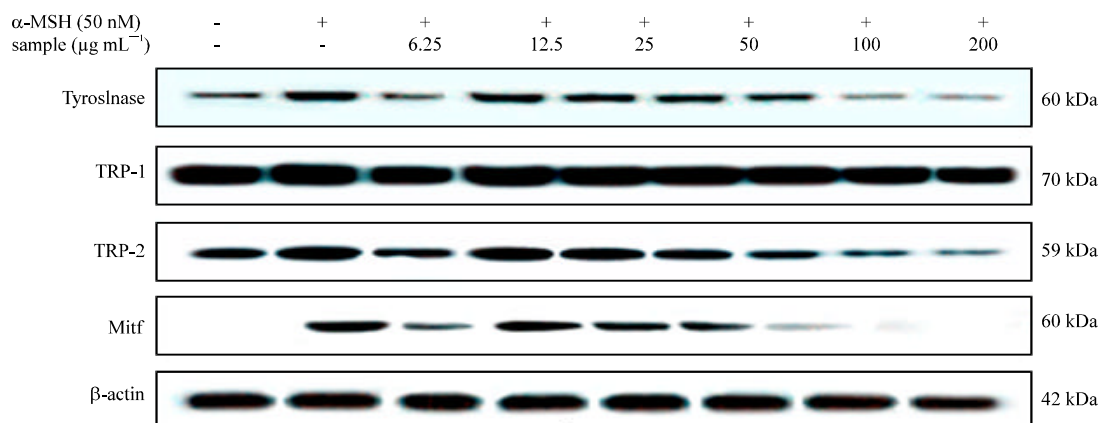


Fig. 3: Inhibitory effect of NAB on tyrosinase, TRP-1, TRP-2, MITF and β -actin protein expression in α -MSH stimulated B16F10 murine melanoma cells. Cells were treated with 50 nM α -MSH in presence or absence of NAB at the indicated concentrations for 72 h, β -actin was served as a loading control, MSH-: negative control without α -MSH, MSH+: positive control with 50nM α -MSH

NAB, its inhibitory mechanism with respect to melanin formation was evaluated. Melanin is one of the heteropolymers produced inside melanosomes by tyrosinase as the key enzyme. It is well documented that, besides tyrosinase, the tyrosinase-like enzymes (TRP-1 and TRP-2) also affect the production of melanin. These proteins constitute a specific family of membrane proteins which are structurally related but have distinct enzymatic functions (Abbott *et al.*, 1991; Kobayashi *et al.*, 1994; Yokoyama *et al.*, 1994). In order to investigate whether NAB can influence melanogenic protein expressions, Western blotting analysis was carried out using the lysate of B16F10 murine melanoma cells stimulated by α -MSH (50 nM). The results are shown Fig. 3. When cells were stimulated by α -MSH, a significant increase of protein levels were observed with tyrosinase, TRP-1 and TRP-2 enzymes. As clearly shown in Fig. 3, addition of NAB to the α -MSH-stimulated cells led to the inhibition of tyrosinase and TRP-2 expression in a dose-dependent manner. However, TRP-1 expression showed no significant changes in this experiment. The protein level of β -actin, a housekeeping protein and used as an internal control, also showed no change. Taken together, these observations suggest that NAB reduced the expression of tyrosinase and TRP-2 in a concentration-dependent manner.

While tyrosinase is the key enzyme in the formation of melanin, TRP-1 and TRP-2 are also involved in melanin synthesis. TRP-1 and TRP-2 are transmembrane protein spanning melanosomal membranes. In murine pigment cells, TRP-1 has been reported to display tyrosinase-like activity. In addition, in mouse melanocytes, TRP-1 is known to influence tyrosinase activity by forming a complex and/or stabilizing tyrosinase (Tief *et al.*, 1996;

Wu and Park, 2003). Nevertheless, in the present study, it is interesting to note that NAB did not have an effect on TRP-1.

On the other hand, NAB substantially reduced the TRP-2 production in B16 melanoma cells. TRP-2 functions as a dopachrome tautomerase in the melanogenic pathway. This enzyme is also known to control the quantity and the quality of the melanin production (Tsukamoto *et al.*, 1992). The TRP-2 gene was also reported to be related to the cytotoxic effects in melanocytes (Huang *et al.*, 2008).

MITF is a factor that effectively transactivates the tyrosinase, TRP-1 and TRP-2 genes i.e., it is considered as a key regulator of melanocyte development. Therefore, the effect of NAB on MITF expression was evaluated. As shown in Fig. 3, based on the Western analysis, the upstream transcription factor MITF was down-regulated in a dose-dependent manner. The MITF is a known specific transcription factor of the tyrosinase gene family (Fuse *et al.*, 1996; Udonio *et al.*, 2000). It is known that the down-regulation of MITF may affect the expressions of all tyrosinase-like genes including tyrosinase, TRP-1 and TRP-2. As MITF protein levels are reduced in this study, the hypopigmentation effect of NAB may be the result of down-regulation of MITF gene expression, which would then repress both the protein and gene expressions of tyrosinase and TRP-2.

CONCLUSION

In the present study, NAB showed potent mushroom tyrosinase inhibitory activity. It also inhibited tyrosinase and TRP-2 activity at the protein levels in melanocytes. Melanogenesis is regulated by a series of enzymes under

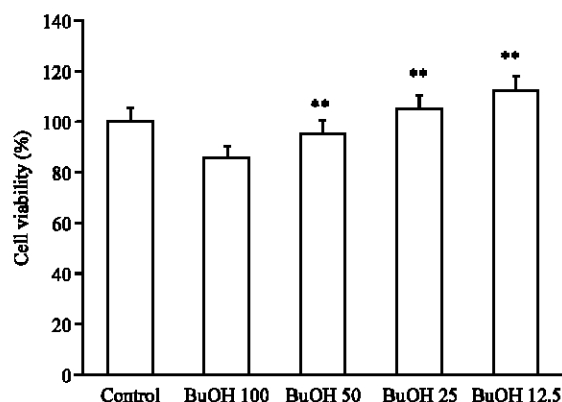


Fig. 4: Effects of NAB on cytotoxicity in cultured human normal fibroblast cell. Cytotoxicities were determined using the MTT method. Values are the Mean \pm SEM of triplicate experiments. **p = 0.01

the control of MITF. In the present study, NAB also reduced MITF production. Some tyrosinase inhibitors isolated from plants have been found to suppress melanogenesis and to have application in prevention and/or treatment of hyperpigmentation. The NAB may prove to be an effective hyperpigmentation or whitening agent that could be used in skin care cosmetics. In order to apply NAB as a topical agent, the NAB should not induce cytotoxic effects on human skin cells. Therefore, we examined the cytotoxic effects of NAB on cultured human dermal fibroblast cells. As shown in Fig. 4, approximately 100% of human dermal fibroblast cell was viable when incubated with 50 $\mu\text{g mL}^{-1}$ of NAB. This strongly suggests its relatively low cytotoxicity. Further investigations will focus on the *in vivo* assessment of the biological activity of NAB and on the chemical identification of the major active components responsible for anti-melanogenesis activity in this extracts. To the best of our knowledge, this is the first report demonstrating the *in vitro* anti-melanogenesis activity of NAB and providing a scientific basis for its cosmetic application.

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