Inhibitory Effects of *Morinda citrifolia* Extract and Its Constituents on Melanogenesis in Murine B16 Melanoma Cells

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Received August 8, 2011; accepted October 20, 2011; published online October 26, 2011

The objective of this study was to examine the effects of *Morinda citrifolia* (noni) extract and its constituents on α-melanocyte stimulating hormone (α-MSH)-stimulated melanogenesis in cultured murine B16 melanoma cells (B16 cells). A 50% ethanolic extract of noni seeds (MCS-ext) showed significant inhibition of melanogenesis with no effect on cell proliferation. MCS-ext was more active than noni leaf and fruit flesh extracts. Activity guided fractionation of MCS-ext led to the isolation of two lignans, 3,3′-bisdemethylpinoresinol (1) and americanin A (2), as active constituents. To elucidate the mechanism of melanogenesis inhibition by the lignans, α-MSH-stimulated B16 cells were treated with 1 (5 μM) and 2 (200 μM). Time-dependent increases of intracellular melanin content and tyrosinase activity, during 24 to 72 h, were inhibited significantly by treatment with the lignans. The activity of 1 was greater than that of 2. Western blot analysis suggested that the lignans inhibited melanogenesis by down regulation of the levels of phosphorylation of p38 mitogen-activated protein kinase, resulting in suppression of tyrosinase expression.

Key words *Morinda citrifolia*; noni seed; B16 melanoma cell; tyrosinase; 3,3′-bisdemethylpinoresinol; americanin A

Melanogenesis is a multistage process involving melanin synthesis, melanin transport, and melanosome release. Tyrosinase is one of the key enzymes in the melanin biosynthetic pathway. Abnormal deposition of melanin pigment causes hyperpigmentary disorders, such as melasma, freckles and age spots. Tyrosinase inhibitor is one of the candidates for reduction of melanogenesis.[1,2] In the development of novel and useful cosmetic agents and functional foods, we have continued to research melanin hyperpigmentation inhibitors from natural sources. The fruit, roots, bark and leaves of a tropical tree, *Morinda citrifolia* L. (Rubiaceae), commonly known as "noni" in Hawaii and Tahiti, have long been used throughout Polynesia as a folk medicine in the treatment of many diseases, *e.g.* hypertension and diabetes.[3] Recently, the noni fruit juice and tea made from noni leaves have been introduced into the functional food market. Noni fruit contains a large number of seed throughout its flesh. During the production of noni fruit juice, these seeds are removed and discarded. Consequently, we have investigated the utility of noni seeds. In a previous paper,[4] we reported the tyrosinase inhibitory activity and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity of 50% ethanolic extracts of noni seeds, fruit flesh and leaves. A 50% ethanolic extract from noni seeds (MCS-ext) inhibited tyrosinase activity and scavenged the DPPH radical more potently than extracts from the leaves or fruit. Activity-guided fractionation, followed by chromatography of MCS-ext, led to the isolation of 3,3′-bisdemethylpinoresinol (**1**), americanin A (**2**) and quercetin (**3**), as active constituents with both tyrosinase inhibitory and radical scavenging activities.[4] After the publication of our previous paper, Akihisa *et al.* reported melanogenesis inhibitory activities of a methanolic extract from noni fruits.[5] However, the effect of noni seed extract (MCS-ext) on melanogenesis in B16 melanoma cells is hitherto unknown. In this paper, we examined the anti-hyperpigmentation activity of MCS-ext and its constituents by α-melanocyte stimulating hormone (α-MSH)-stimulated melanogenesis in cultured murine B16 melanoma cells (B16 cells).

MATERIALS AND METHODS

Plant Materials Fruits and leaves of *M. citrifolia* were collected in French Polynesia during 2004—2006. These samples were identified by Tropical Resources, Inc. (Provo, UT, U.S.A.). Fruit samples were separated into flesh and seeds by hand. The fruit flesh was freeze-dried. The leaves and seeds were air-dried. Voucher specimens of dried noni fruit flesh (Noni: Lot Code. 2555900), leaves (Noni Leaf: L06-017-MIT 75) and seeds (Noni Seed: L06-017-MIT75) are deposited in Kinki University.

Preparation and Fractionation of 50% Ethanolic Extract from Noni The preparation of 50% ethanolic extracts of dried flesh (MCF-ext, yield 53%), dried leaves (MCL-ext, yield 36%) and dried seeds (MCS-ext, yield 5%), have been described previously.[4] A suspension of the MCS-ext (30 g) in water (120 mL) was extracted with hexane (120 mL×2) followed by ethyl acetate (120 mL×10). Evaporation of solvent from each combined extract gave a hexane-soluble fraction (0.29 g) and an ethyl acetate-soluble fraction (5.4 g). The aqueous layer was evaporated and then followed by lyophilization to give a water-soluble fraction (22 g).

Reagents The isolation of lignans (**1**, **2**) from MCS-ext has also been described previously.[4] Quercetin (**3**) and ursolic acid (**4**) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). 3,4-Dihydroxy-L-phenylalanine (L-dopa), [Nle[4], D-Phe[7]]-α-melanocyte stimulating hormone trifluoroacetate salt (α-MSH) and synthetic melanin were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Protease inhibitor mixture was purchased from Calbiochem (Darmstadt, Germany). The antibody to tyrosinase was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Antibodies against p38 mitogen-activated protein kinase (p38 MAPK), extracellular signal-regulated kinase 1/2 (ERK1/2), phosphor-

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specific p70 ribosomal S6 kinase (p70 S6K), phosphor-specific p38 MAPK, phosphor-specific ERK1/2 and β-actin were purchased from Cell Signaling Technology (Beverly, MA, U.S.A.). The enhanced chemiluminescence (ECL) Plus Western blot detection kit and ECL anti-rabbit immunoglobulin G (IgG), horseradish peroxidase linked spices-specific whole antibody were obtained from GE Healthcare U.K. Ltd. (Little Chalfont, England). Other chemical and biochemical reagents were of reagent grade and were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and/or Nacalai Tesque, Inc. (Kyoto, Japan) unless otherwise noted.

Cell Culture The B16 cells (B16F1) were purchased from Dainippon Sumitomo Pharmaceutical Co., Ltd. (Osaka, Japan) in Oct. 2009. The B16 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich) supplemented with 10% (v/v) fetal bovine serum (Nichirei Biosciences Inc., Tokyo, Japan) and 1% antibiotic–antimycotic solution (a mixture of 10000 U/mL penicillin, 10000 μg/mL streptomycin sulfate and 25 μg/mL amphotericin B, Invitrogen Corp., Carlsbad, CA, U.S.A.) at 37°C in a humidified, CO_2-controlled (5%) incubator. Stimulation of melanogenesis was initiated by the addition of α-MSH.

Melanogenic Assays The amount of intracellular melanin in B16 cells was measured at the indicated times after treatment of samples according to the method of Ohguchi *et al.*[6] Briefly, cells (2×10^4 cells) were seeded on 24-well plates with 800 μL and treated with test samples (100 μL) and α-MSH (100 μL) at 24 h after seeding. Test samples were dissolved in dimethyl sulfoxide (DMSO) and then diluted with DMEM to an appropriate concentration. The final concentration of DMSO was 0.1% v/v. In the control and vehicle control groups, DMSO solution was used instead of the sample solution. α-MSH was added to the medium in the vehicle control and test groups. α-MSH was dissolved in aqueous acetic acid solution (5%, v/v) and then diluted with DMEM to 1 μM of the final concentration. The mixture was incubated at 37°C for the indicated times. The B16 cells were washed twice with phosphate-buffered saline (PBS, pH 7.0) and dissolved in 2 N NaOH for 1 h at 65°C. The absorbance at 490 nm of each sample was measured by using a microplate reader (Sunrise Rainbow Thermo, Tecan, Männedorf, Switzerland) and melanin amount was determined by using the authentic standard of synthetic melanin. The cell proliferation of B16 cells was assessed according to the method as described previously.[7] Cell proliferation was shown in percentages. Each percentage in the treated cells was calculated with respect to that of the vehicle control cells.

Tyrosinase Activity Intracellular tyrosinase activity was measured at the indicated times after treatment of samples according to the method of Ohguchi *et al.*[6] The cells were washed twice with ice-cold PBS and then lysed by incubating at 4°C for 30 min in a lysis buffer (2 mM Tris–HCl, pH 7.5, 15 mM NaCl, 0.1 mM disodium salt of ethylenediaminetetraacetic acid, 0.1% triton, and 0.1 mM ethylene glycol tetraacetic acid, Cell Signaling Technology) containing protease inhibitors. The lysates were centrifuged at 15000×***g***, 4°C, for 20 min to obtain a supernatant as source of tyrosinase. The reaction mixture, containing 50 mM phosphate buffer (pH 7.4), 0.05% L-dopa and the supernatant (tyrosinase), was incubated at 37°C for 20 min. After incubation, dopachrome formation was assayed by measuring absorbance at 475 nm with a microplate reader. Tyrosinase activity was shown in percentages. Each percentage in the treated cells was calculated with respect to that in the pretreated cells.

Electrophoresis and Western Blot Analysis According to the method of Ohguchi *et al.*,[6] B16 cells were lysed in a lysis buffer containing protease inhibitors and centrifuged at 15000×***g***, 4°C, for 20 min. The resulting supernatant (solubilized proteins) was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 7.5% polyacrylamide gel. Proteins were transferred electrophoretically onto a polyvinylidene-difluoride (PVDF) membrane. After blocking in 5% skimed milk in PBS containing 0.05% Tween 20, PVDF membranes were incubated with an appropriate primary antibody and then further incubated with horseradish peroxidase-conjugated secondary antibody. Proteins were visualized using the ECL Plus Western blot detection kit. Gel images were obtained with a ChemiDoc XRS imaging system (Bio-Rad Laboratories, Inc., Hercules, CA, U.S.A.).

Statistical Analysis The experimental data were evaluated for statistical significance by Bonferroni/Dunn's multiple range test with Stat View for Windows, Ver. 5 (SAS Institute Inc., 1998).

RESULTS AND DISCUSSION

Effect of Noni Extracts on Melanogenesis in α-MSH-Stimulated B16 Cells The cultured murine B16 melanoma cells (B16 cells) have been frequently used for *in vitro* melanogenesis assay. Melanogenesis is stimulated by various effectors, including an α-melanocyte stimulating hormone (α-MSH), theophylline,[8] cAMP elevating agents (*e.g.*, forskolin,[9] isobutylmethylxanthine[10] and glycyrrhizin[11]) and UV light. Among the B16 cell stimulators, α-MSH has been the most frequently used in studies of melanogenesis regulators.[6] In the preliminarily tests, melanogenesis stimulation by α-MSH in B16 cells was observed at a concentration of 1 μM of α-MSH, but not at 10 nM and 100 nM (data not shown) as described by Smalley *et al.*[12] Thus, we used α-MSH (1 μM) as a melanogenesis stimulator in our experiments as reported by Ohguchi *et al.*[6]

The α-MSH-stimulated B16 cells were treated with MCF-ext, MCL-ext and MCS-ext for 72 h. Inhibition of melanogenesis was evaluated by measurement of the intracellular melanin content which is shown in μg/well. Cell proliferation was expressed as a percentage of the controls. As shown in Table 1, MCS-ext (12.5 to 200 μg/mL) and kojic acid (100 to 200 μM), a reference compound which is a well known melanogenesis inhibitor with potent tyrosinase inhibitory activity (IC_{50} value: 30 μM[4]), inhibited α-MSH (1 μM)-simulated melanin production in a concentration-dependent manner without any significant effects on cell proliferation, whereas MCF-ext and MCL-ext were inactive at 200 μg/mL. In contrast, Akihisa *et al.*[5] reported that a methanolic extract of noni fruits at 100 μg/mL inhibited α-MSH (100 nM)-stimulated melanogenesis in B16 cells. They also described that a number of iridoid-, hemiterpene- and fatty acid-glycosides from the methanolic extract of noni fruits exhibited melanogenesis inhibitory activities at a concentration of 100 μM.[5]

Inhibition of Melanogenesis in α-MSH-Stimulated B16 Cells by 3,3′-Bisdemethylpinoresinol (1), Americanin A

Table 1. Effects of MCF-ext, MCL-ext and MCS-ext on α-MSH-Stimulated Melanogenesis in B16 Cells

Samples	Concentration (μg/mL or μM)	α-MSH (final conc.)	Melanin content (μg/well)	Cell proliferation (%)
Control			1.6±0.4	61.9±1.2
Vehicle control		1 μM	42.8±1.6##	100.0±1.2##
MCF-ext	12.5 (μg/mL)	1 μM	39.3±2.1	99.7±0.5
	50	1 μM	38.2±1.6	100.0±2.3
	200	1 μM	41.3±1.0	96.7±0.9
MCL-ext	12.5 (μg/mL)	1 μM	41.7±1.2	100.6±1.7
	50	1 μM	43.7±0.4	101.6±2.9
	200	1 μM	38.9±0.8	102.0±1.5
MCS-ext	12.5 (μg/mL)	1 μM	29.0±0.6**	100.6±1.1
	50	1 μM	21.1±0.6**	105.9±1.2
	200	1 μM	11.9±0.6**	103.5±2.5
Kojic acid	100 (μM)	1 μM	13.5±0.4**	107.4±1.9
	200	1 μM	6.9±0.4**	103.2±2.4

Each value in melanin content represents the mean±S.E. of 3 experiments. ##$p<0.01$: statistically significant *vs.* the value of control group. **$p<0.01$: statistically significant *vs.* the value of vehicle control group. Each value in cell proliferation represents the mean±S.E. of 3 experiments.

Table 2. Effects of Hexane-sol. Fr., Ethyl Acetate-sol. Fr. and Water-sol. Fr. from MCS-ext on α-MSH-Stimulated Melanogenesis in B16 Cells

Samples	Concentration (μg/mL or μM)	α-MSH (final conc.)	Melanin content (μg/well)	Cell proliferation (%)
Control			1.6±1.6	60.2±5.7
Vehicle control		1 μM	36.4±2.5##	100.0±1.5##
Hexane-sol. Fr.	12.5 (μg/mL)	1 μM	29.3±1.9	96.6±2.3
	50	1 μM	19.8±2.2**	100.5±3.9
Ethyl acetate-sol. Fr.	12.5 (μg/mL)	1 μM	24.9±3.0**	95.2±1.4
	50	1 μM	17.2±0.6**	104.1±0.8
Water-sol. Fr.	12.5 (μg/mL)	1 μM	43.5±2.5	99.1±3.4
	50	1 μM	39.1±3.7	102.3±3.1
Kojic acid	100 (μM)	1 μM	18.1±0.6**	102.2±0.5
	200	1 μM	8.4±1.2**	103.9±1.4

Each solvent sol. Fr. means each solvent soluble fraction. Each value in melanin content represents the mean±S.E. of 3 experiments. ##$p<0.01$: statistically significant *vs.* the value of control group. **$p<0.01$: statistically significant *vs.* the value of vehicle control group. Each value in cell proliferation represents the mean±S.E. of 3 experiments.

(2), Quercetin (3) and Ursolic Acid (4) Activity guided fractionation of MCS-ext with α-MSH-stimulated B16 cells was carried out to identify the active constituents of MCS-ext. Fractionation of MCS-ext by solvent extraction gave three fractions, namely a hexane-soluble fraction (yield from MCS-ext: 1%), an ethyl acetate-soluble fraction (18%) and a water-soluble fraction (73%). At concentrations ranging from 12.5 to 50 μg/mL, the ethyl acetate-soluble fraction exhibited more potent activity than the hexane-soluble fraction without any significant effects on cell proliferation, as described in Table 2. The water-soluble fraction was inactive. As to the constituents of the ethyl acetate-soluble fraction of MCS-ext, the isolation of **1** (isolation yield from MCS-ext: 1.17%; isolation yield from dried seeds: 0.055%), **2** (0.51%; 0.024%), **3** (0.19%; 0.009%) and **4** (0.74%; 0.035%) from this fraction has been reported in the previous paper.[4] The IC_{50} values of tyrosinase inhibitory activities of **1**, **2** and **3** were 0.3, 2.7 and 0.1 mM, respectively, whereas **4** was inactive.[4] To identify active constituents with melanogenesis inhibition in α-MSH-stimulated B16 cells, the activities of **1**, **2**, **3** and **4** were assayed. As shown in Table 3, a lignan, **1**, displayed potent inhibitory activity on α-MSH-stimulated melanin production. Inhibition by **1** was concentration-dependent from 1.25 to 5 μM without any significant effects on cell proliferation. However, **1** decreased cell proliferation at high concentrations (10, 20 μM). Another lignan, **2**, also inhibited melanin production at concentrations of 100 to 200 μM without any significant effects on cell proliferation.

Although **3** exhibited potent tyrosinase inhibitory activity, it had no effect on melanin content and cell proliferation at concentrations of 12.5 to 200 μM. This fact was not in accordance with the report in which **3** enhanced melanogenesis in human HMVII melanoma cells at a concentration of 20 μM and in normal human epidermal melanocytes at a concentration of 1 μM.[13] Moreover, the influence of **3** on melanogenesis is controversial. Recently, Yang *et al.*[14] suggested that **3** had different and opposite effects on melanogenesis depending on the experimental conditions, *e.g.* concentrations of **3** and species of melanoma cells. Compound **4**, with no tyrosinase inhibitory activity, was inactive on B16 cells. Considering with the content of **1** and **2** in MCS-ext, the melanogenesis inhibitory activity of MCS-ext may be attributable to the two lignans.

Inhibition of Tyrosinase Activity in α-MSH-Stimulated B16 Cells by 3,3′-Bisdemethylpinoresinol (1) and Americanin A (2) Since **1** and **2** are newly described as melanogenesis inhibitors, we attempted to elucidate their melanogenesis suppression mechanism. The α-MSH-stimulated B16 cells were treated with **1** and **2** at a concentration of 5 and 200 μM, respectively, as these concentrations were found to be the most effective in the previous assay (Table 3).

The time-course of intracellular melanin content and ty-

Table 3. Effects of 3,3′-Bisdemethylpinoresinol, Americanin A, Quercetin and Ursolic Acid on α-MSH-Stimulated Melanogenesis in B16 Cells

Samples	Concentration (μM)	α-MSH (final conc.)	Melanin content (μg/well)	Cell proliferation (%)
Run 1				
Control			4.5±0.8	55.2±1.3
Vehicle control		1 μM	37.5±1.5##	100.0±1.1##
3,3′-Bisdemethylpinoresinol (**1**)	1.25 (μM)	1 μM	29.9±0.6**	97.8±0.4
	2.5	1 μM	21.6±0.8**	101.0±1.7
	5	1 μM	18.5±1.2**	99.1±0.7
	10	1 μM	15.2±0.2**	88.8±1.2**
	20	1 μM	13.2±0.8**	88.5±0.3**
Kojic acid	100 (μM)	1 μM	17.2±1.1**	97.1±1.3
	200	1 μM	6.4±0.4**	98.2±1.4
Run 2				
Control			7.3±2.5	58.8±2.4
Vehicle control		1 μM	39.0±0.9##	100.0±5.1##
Americanin A (**2**)	12.5 (μM)	1 μM	39.3±0.7	101.6±1.0
	25	1 μM	36.7±0.7	107.1±0.4
	50	1 μM	33.9±0.8	103.3±0.9
	100	1 μM	25.5±1.5**	99.4±4.5
	200	1 μM	13.9±1.0**	102.8±1.4
Quercetin (**3**)	12.5 (μM)	1 μM	41.7±0.6	100.6±10.1
	25	1 μM	43.0±2.0	95.8±2.8
	50	1 μM	44.6±0.4	91.3±4.4
	100	1 μM	36.5±7.1	103.6±6.3
	200	1 μM	40.0±1.2	100.2±4.0
Ursolic acid (**4**)	12.5 (μM)	1 μM	36.7±0.7	101.8±7.9
	25	1 μM	40.2±0.4	100.5±2.9
	50	1 μM	41.1±0.8	100.6±2.6
	100	1 μM	40.4±2.5	108.0±5.6
	200	1 μM	42.4±2.3	108.6±5.7
Kojic acid	100 (μM)	1 μM	15.7±1.0**	101.9±3.8
	200	1 μM	8.6±0.6**	98.4±2.7

Each value in melanin content represents the mean±S.E. of 3 experiments. ##$p<0.01$: statistically significant *vs.* the value of control group. **$p<0.01$: statistically significant *vs.* the value of vehicle control group. Each value in cell proliferation represents the mean±S.E. of 3 experiments.

rosinase activities after treatment of α-MSH-stimulated B16 cells with **1** and **2** are depicted in Fig. 1. Each percentage of tyrosinase activity in the treated cells was calculated with respect to the activity in pre-treated cells. As shown in Fig. 1A, the intracellular melanin content of the control group time-dependently increased from 24 to 72 h, whereas the melanin content of the treated group decreased significantly 72 h after addition of the lignans. As shown in Fig. 1B, the time-dependent increase of tyrosinase activity from 24 to 72 h was also inhibited significantly by treatment with the lignans.

Effect of 3,3′-Bisdemethylpinoresinol (1) and Americanin A (2) on Tyrosinase Expression in α-MSH-Stimulated B16 Cells To further investigate the anti-melanogenesis mechanisms of **1** and **2**, we used Western blot analysis to assess the levels of tyrosinase expression in α-MSH-stimulated B16 cells. As shown in Fig. 2, the tyrosinase expression at 72 h was enhanced remarkably by the stimulation with α-MSH. After the treatment of the α-MSH-stimulated B16 cells with **1** (5 μM) and **2** (200 μM), the tyrosinase expression did not increase at 72 h. Thus, inhibition of melanogenesis by the lignans was associated with a corresponding decrease of tyrosinase expression and consequent activity.

Effect of 3,3′-Bisdemethylpinoresinol (1) and Americanin A (2) on Phosphorylation of p38 MAPK in α-MSH-Stimulated B16 Cells During melanogenesis in melanocytes, the p38 MAPK cascade activates microphthalmia-associated transcription factor (MITF), which positively regulates tyrosinase gene expression, whereas p70 S6K and ERK1/2 cascades depress MITF.[15,16] Recently, the p38 MAPK cascade was demonstrated to be involved in the melanogenesis in B16 cells induced by α-MSH.[12] More recently, negative regulation of melanogenesis by phospholipase D1 through mammalian target of rapamycin (mTOR)/p70 S6K signaling in B16 cells has been reported.[17] In the preliminarily time-course studies on the levels of phosphorylation of p38 MAPK in α-MSH (1 μM)-stimulated B16 cells at 0, 1, 2, 3, 4, 5, 6 and 12 h, a significant increase of the phosphorylation level was detected at first at 6 h and the highest increment was observed at 12 h as shown in Fig. 3A (other data not shown) in contrast to the reported transient peak at 1 h in α-MSH (10 nM)-stimulated B16 cells.[12] We assumed that a part of the discrepancy of the peak time might be due to the differences in experimental conditions, such as B16 cell lines and concentrations of α-MSH. The effects of **1** (5 μM) and **2** (200 μM) on phosphorylation levels of p38 MAPK, ERK1/2 and p70 S6K in α-MSH (1 μM)-stimulated B16 cells were examined by Western blot analysis using the respective mouse antibody for phosphorylated forms of p38 MAPK, ERK1/2 and p70 S6K. As shown in Fig. 3A, a time-course analysis at 6 and 12 h revealed that the levels of phosphorylation of p38 MAPK in α-MSH-stimulated B16 cells were enhanced by α-MSH treatment in comparison to those without α-MSH. Smalley *et al.*[12] reported

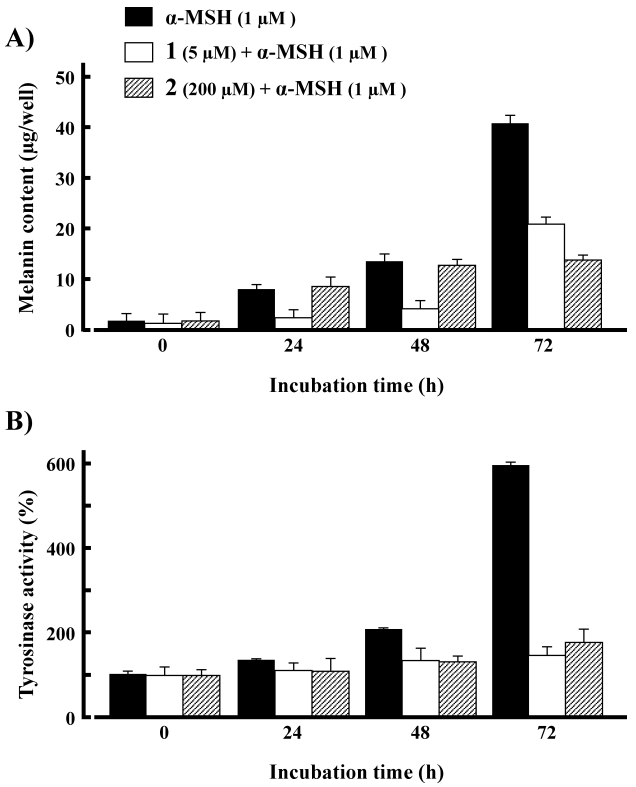

Fig. 1. Effects of 3,3′-Bisdemethylpinoresinol (**1**) and Americanin A (**2**) on Melanogenesis and Tyrosinase Activation in α-MSH-Stimulated B16 Cells

The cells were treated with α-MSH (1 μM, black column), **1** (5 μM, white column) and **2** (200 μM, slashed column) for the indicated times. (A) The melanin content was determined as described under Materials and Methods. (B) Tyrosinase activity was determined by measuring the formation of dopachrome as described under Materials and Methods. Data represent means±S.E. of two different experiments each carried out in triplicate.

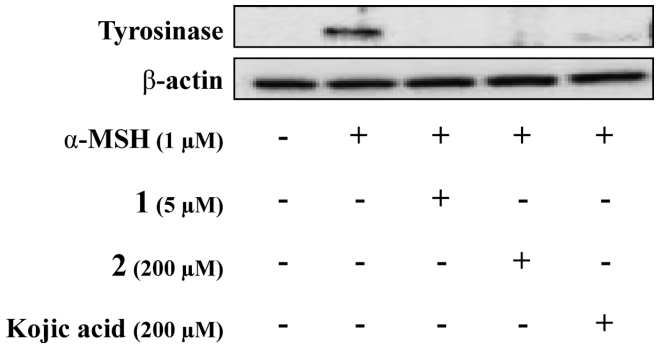

Fig. 2. Effects of 3,3′-Bisdemethylpinoresinol (**1**), Americanin A (**2**) and Kojic Acid on Tyrosinase Expression in α-MSH-Stimulated B16 Cells

The cells were treated with α-MSH (1 μM) in the presence of **1** (5 μM), **2** (200 μM) or kojic acid (200 μM) for 72 h. The level of tyrosinase expression was examined by Western blot analysis using specific antibody as described under Materials and Methods. Equal protein loading was confirmed by β-actin expression.

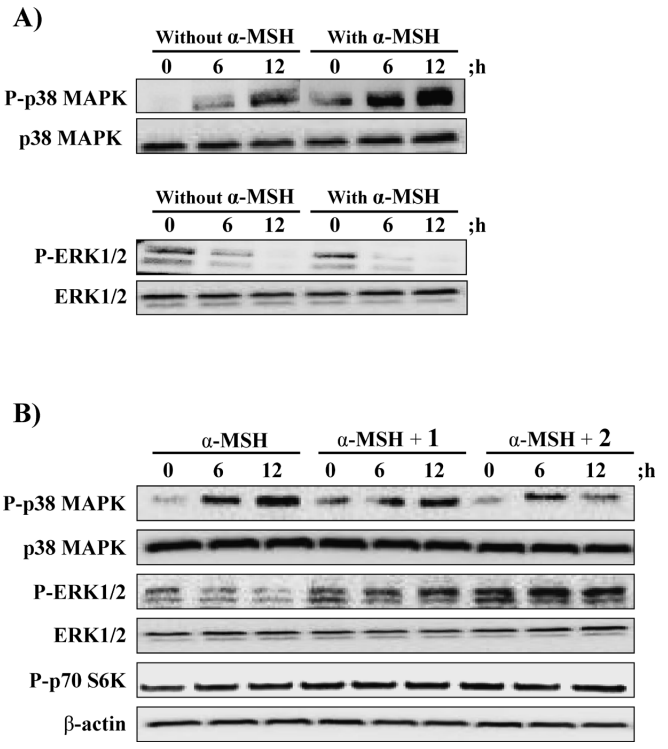

Fig. 3. (A) Effects of α-MSH on Phosphorylation of p38 MAPK and ERK1/2 in B16 Cells. (B) Effects of 3,3′-Bisdemethylpinoresinol (**1**) and Americanin A (**2**) on Phosphorylation of p38 MAPK, ERK1/2 and p70 S6K in α-MSH-Stimulated B16 Cells

A) The cells were treated with or without α-MSH (1 μM) for the indicated times. B) The cells were treated with α-MSH (1 μM) in the presence of **1** (5 μM) or **2** (200 μM) for the indicated times. Phosphorylation of p38 MAPK, ERK1/2 and p70 S6K was assessed by Western blot analysis with using the mouse specific antibody for phosphorylated forms of p38 MAPK, ERK1/2 and p70 S6K. Equal protein loading was confirmed by β-actin expression.

that the levels of ERK1/2 phosphorylation in B16 cells were enhanced by α-MSH (10 nM) treatment. In contrast, according to the paper by Kumar *et al.*,[18] the level of phosphorylation was not affected in the presence of α-MSH (100 nM). In our experiment, phosphorylation was suppressed time-dependently at 6 and 12 h, regardless of the presence or absence of α-MSH (Fig. 3A). Treatment with **1** and **2** both suppressed α-MSH-stimulated p38 MAPK phosphorylation and enhanced ERK1/2 phosphorylation at 6 and 12 h (Fig. 3B). The lignans had no effect on p70 S6K phosphorylation (Fig. 3B). These results indicate that the lignans may decrease the phosphorylation of p38 MAPK, whose cascade activates MITF. The lignans may also elevate phosphorylation of ERK1/2, whose cascade depresses MITF.

It has been reported that deoxypodophyllotoxin, a lignan with anti-inflammatory and anti-atherosclerosis activities, inhibited matrix metalloproteinase-9 which plays key role in atherosclerosis *via* MAPK pathways in tumor necrosis factor-α stimulated human aortic smooth muscle cells, namely deoxypodophyllotoxin inhibited the phosphorylation of p38 MAPK, ERK1/2 and c-Jun-N-terminal kinase in the smooth muscle cells.[19] It was found for the first time that the lignans, **1** and **2**, inhibited tyrosinase expression *via* inhibition of p38 MAPK phosphorylation and elevation of ERK1/2 phosphorylation. The tyrosinase inhibitory activity of **1** (IC_{50} value: 0.3 mM)[4] was comparatively weak, whereas **1** showed a potent anti-melanogenesis in α-MSH stimulated B16 cells at lower concentrations of 1.25 to 5 μM. It may be considered that the inhibition of tyrosinase expression was contributory to the potent anti-melanogenesis activity.

In conclusion, MCS-ext significantly inhibited melanogenesis in α-MSH-stimulated B16 cells, without any effects on cell proliferation. The lignans, **1** and **2**, isolated from MCS-ext decreased intracellular tyrosinase activity and melanin content. Western blot analysis suggested that the lignans inhibited mel-

anogenesis through the suppression of tyrosinase expression, resulting from down regulation of p38 MAPK phosphorylation and elevation of ERK1/2 phosphorylation. The melanogenesis inhibitory activity of MCS-ext in α-MSH-stimulated B16 cells was confirmed, along with previously reported tyrosinase inhibitory and radical scavenging activities. Thus MCS-ext may be a useful ingredient for the prevention of hyperpigmentation.

Acknowledgments We thank Prof. Kenji Ohguchi, the Department of Food and Nutrition, Sugiyama Jogakuen University, for his invaluable technical advice. This work was financially supported by "Antiaging Center Project" for Private Universities from the Ministry of Education, Culture, Sports, Science and Technology of Japan, 2008—2012.

REFERENCES

1) Solano F, Briganti S, Picardo M, Ghanem G. Hypopigmenting agents: an updated review on biological, chemical and clinical aspects. *Pigment Cell Res.*, **19**, 550—571 (2006).
2) Parvez S, Kang M, Chung HS, Cho C, Hong MC, Shin MK, Bae H. Survey and mechanism of skin depigmenting and lightening agents. *Phytother. Res.*, **20**, 921—934 (2006).
3) Ross IA. *Morinda citrifolia*. "Medicinal plants of the world," Vol. II, Chap. 16, Humana Press, Totowa, 2001, pp. 309—317.
4) Masuda M, Murata K, Fukuhama A, Naruto S, Fujita T, Uwaya A, Isami F, Matsuda H. Inhibitory effects of constituents of *Morinda citrifolia* seeds on elastase and tyrosinase. *J. Nat. Med.*, **63**, 267—273 (2009).
5) Akihisa T, Seino K, Kaneko E, Watanabe K, Tochizawa S, Fukatsu M, Banno N, Metori K, Kimura Y. Melanogenesis inhibitory activities of iridoid-, hemiterpene-, and fatty acid-glycosides from the fruits of *Morinda citrifolia* (Noni). *J. Oleo Sci.*, **59**, 49—57 (2010).
6) Ohguchi K, Akao Y, Nozawa Y. Involvement of calpain in melanogenesis of mouse B16 melanoma cells. *Mol. Cell. Biochem.*, **275**, 103—107 (2005).
7) Matsuda H, Kawaguchi Y, Yamazaki M, Hirata N, Naruto S, Asanuma Y, Kaihatsu T, Kubo M. Melanogenesis stimulation in murine B16 melanoma cells by *Piper nigrum* leaf extract and its lignan constituents. *Biol. Pharm. Bull.*, **27**, 1611—1616 (2004).
8) Hu F. Theophylline and melanocyte-stimulating hormone effects on gamma-glutamyl transpeptidase and DOPA reactions in cultured melanoma cells. *J. Invest. Dermatol.*, **79**, 57—62 (1982).
9) Huh S, Kim YS, Jung E, Lim J, Jung KS, Kim MO, Lee J, Park D. Melanogenesis inhibitory effect of fatty acid alkyl esters isolated from *Oxalis triangularis*. *Biol. Pharm. Bull.*, **33**, 1242—1245 (2010).
10) Englaro W, Rezzonico R, Durand-Clément M, Lallemand D, Ortonne JP, Ballotti R. Mitogen-activated protein kinase pathway and AP-1 are activated during cAMP-induced melanogenesis in B-16 melanoma cells. *J. Biol. Chem.*, **270**, 24315—24320 (1995).
11) Lee J, Jung E, Park J, Jung K, Park E, Kim J, Hong S, Park J, Park S, Lee S, Park D. Glycyrrhizin induces melanogenesis by elevating a cAMP level in B16 melanoma cells. *J. Invest. Dermatol.*, **124**, 405—411 (2005).
12) Smalley K, Eisen T. The involvement of p38 mitogen-activated protein kinase in the α-melanocyte stimulating hormone (α-MSH)-induced melanogenic and anti-proliferative effects in B16 murine melanoma cells. *FEBS Lett.*, **476**, 198—202 (2000).
13) Nagata H, Takekoshi S, Takeyama R, Homma T, Yoshiyuki Osamura R. Quercetin enhances melanogenesis by increasing the activity and synthesis of tyrosinase in human melanoma cells and in normal human melanocytes. *Pigment Cell Res.*, **17**, 66—73 (2004).
14) Yang YM, Son YO, Lee SA, Jeon YM, Lee JC. Quercetin inhibits α-MSH-stimulated melanogenesis in B16F10 melanoma cells. *Phytother. Res.*, **25**, 1166—1173 (2011).
15) Widlund HR, Fisher DE. *Microphthalamia*-associated transcription factor: a critical regulator of pigment cell development and survival. *Oncogene*, **22**, 3035—3041 (2003).
16) Kim DS, Hwang ES, Lee JE, Kim SY, Kwon SB, Park KC. Sphingosine-1-phosphate decreases melanin synthesis *via* sustained ERK activation and subsequent MITF degradation. *J. Cell Sci.*, **116**, 1699—1706 (2003).
17) Ohguchi K, Banno Y, Nakagawa Y, Akao Y, Nozawa Y. Negative regulation of melanogenesis by phospholipase D1 through mTOR/p70 S6 kinase 1 signaling in mouse B16 melanoma cells. *J. Cell. Physiol.*, **205**, 444—451 (2005).
18) Kumar KJ, Yang JC, Chu FH, Chang ST, Wang SY. Lucidone, a novel melanin inhibitor from the fruit of *Lindera erythrocarpa* Makino. *Phytother. Res.*, **24**, 1158—1165 (2010).
19) Suh SJ, Kim JR, Jin UH, Choi HS, Chang YC, Lee YC, Kim SH, Lee IS, Moon TC, Chang HW, Kim CH. Deoxypodophyllotoxin, flavolignan, from *Anthriscus sylvestris* Hoffm. inhibits migration and MMP-9 *via* MAPK pathways in TNF-α-induced HASMC. *Vascul. Pharmacol.*, **51**, 13—20 (2009).