Melanogenesis Inhibitory and Free Radical Scavenging Activities of Diarylheptanoids and Other Phenolic Compounds from the Bark of *Acer nikoense*

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Melanogenesis inhibitory and free radical scavenging activities of nine cyclic (1—9) and one acyclic diarylheptanoids (10), and two phenolic compounds, (+)-rhododendrol (11) and (+)-catechin (12), isolated from the ethyl acetate-soluble fraction of the MeOH extract of the bark of *Acer nikoense* MAXIM. (Aceraceae) were examined. Upon evaluation of compounds 1—12 on the melanogenesis in the B16 melanoma cells, two compounds, 2 and 8, exhibited marked inhibitory activity with 55.6% and 46.8% reduction, respectively, of melanin content at 25 μg/ml without inhibition of cell proliferation. In addition, upon an evaluation of eleven compounds, 1—7 and 9—12 against the scavenging activities of free radicals (against the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical), compound 12 (IC_{50} 9.0 μM) followed by compounds 1, 3, 4, and 6 (IC_{50} 40.2—44.0 μM) showed potent scavenging activities.

Key words *Acer nikoense*; diarylheptanoid; melanogenesis; antioxidant activity; radical scavenging

The bark from the Japanese maple tree, *Acer nikoense* MAXIM. (Aceraceae) (Japanese name, Megusurino-ki) has been used as a folk medicine for the treatment of hepatic disorders and eye disease.[1)] The bark has been reported to contain various diarylheptanoid and phenolic compounds[2—4)] possessing several biological properties[5,6)] including hepatoprotective effects.[7)] In the course of our studies on the bioactive principles from natural sources, we have evaluated recently the diarylheptanoid and phenolic constituents of the extract of *A. nikoense* stem bark for their inhibitory effects on 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced inflammation in mice, and on Epstein-Barr virus early antigen (EBV-EA) activation induced by TPA.[8)] In this paper, we describe the inhibitory effects against melanogenesis in the B16 melanoma cells and free radical scavenging activities on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical of the extracts and their diarylheptanoid and phenolic constituents of *A. nikoense* bark.

MATERIALS AND METHODS

Instruments Silica gel and C_{18} silica (Chromatorex-ODS; Fuji Silysia Chemical Ltd., Aichi, Japan) were used for open column chromatography. Reversed-phase preparative HPLC was carried out on a C_{18} silica column (25 cm×10 mm i.d.; flow rate of the mobile phase: 3.0 ml/min) at 25 °C.

Materials and Chemicals The stem bark obtained from a 25 year old tree of *Acer nikoense* in the summer of 2002 was purchased from Sirakami Fruit Park (Gunma, Japan).[8)] Fetal bovine serum (FBS) was purchased from Trace Scientific LTD (Melbourne, Australia). Eagle's minimal essential medium (MEM) was obtained from Gibco (Grand Island, NY, U.S.A.). 1,1-Diphenyl-2-picrylhydrazyl (DPPH), arbutin (4-hydroxyphenyl β-D-glucopyranoside), and DL-α-tocopherol were purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan).

Extraction and Isolation Extraction of the bark of *A. nikoense*, and isolation and identification of ten diarylheptanoids, **1**—**10**, and two phenolic compounds, **11** and **12**, were described in our recent paper.[8)] The dried stem bark of *A. nikoense* (450 g) was finely cut and extracted three times with *n*-hexane under reflux for 3 h which yielded the extract (3.4 g). The residue was then extracted three times with MeOH under reflux for 3 h giving the extract (63.6 g) which

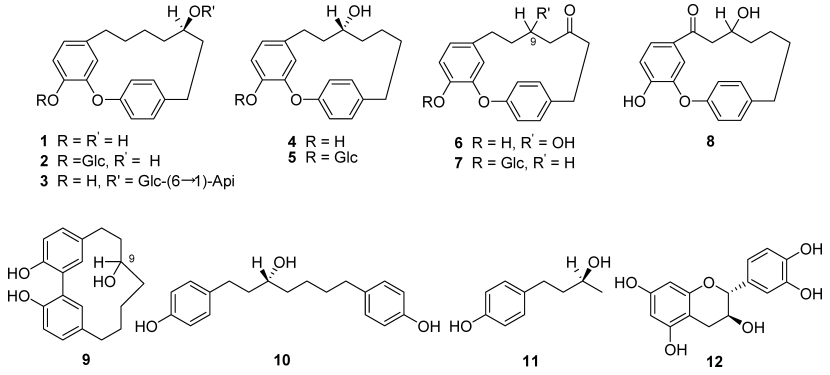

Chart 1. Structures of Compounds **1**—**12**

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was partitioned in an EtOAc–H_2O (1 : 1, v/v) mixture. The aqueous layer was extracted with *n*-butanol (*n*-BuOH), and removal of the solvent from the EtOAc-, *n*-BuOH-, and H_2O-soluble portions yielded 26.2, 26.6, and 4.5 g of the residue, respectively.

Column chromatography on silica gel (800 g) of the EtOAc-soluble fraction (25.2 g) eluted with *n*-hexane–EtOAc (1 : 0→0 : 1) and then with EtOAc–MeOH (7 : 3→0 : 1) gave five fractions, fractions 1—5. Fraction 3 (1.98 g) was further separated by column chromatography on silica gel (100 g) [*n*-hexane–EtOAc (2 : 3→0 : 1) and then EtOAc–MeOH (95 : 5→0 : 1)] to furnish six fractions, fractions 3-1—3-6. Preparative HPLC [MeOH–H_2O–acetic acid (AcOH) (65 : 35 : 0.1, v/v/v)] of fraction 3-1 (140 mg) gave acerogenin A (**1**), (*R*)-acerogenin B (**4**), and (−)-centrolobol (**10**). In addition, preparative HPLC of fraction 3-2 (416 mg) under the same condition as above yielded compounds **1**, **4**, acerogenin D (**6**) (C-9ξ),[3] acerogenin M (**8**) (C-9ξ),[8] acerogenin K (**9**) (C-9*R*/*S* mixture),[4] and **10**, along with a highly polar fraction. The latter fraction was further subjected to HPLC [MeOH–H_2O–AcOH (50 : 50 : 0.1, v/v/v)] which afforded (+)-rhododendrol (**11**). Chromatography of fraction 4 (528 mg) on ODS column (45 g) [MeOH–H_2O (1 : 4→1 : 0)] gave four fractions, fractions 4-1—4-4. Upon HPLC [acetonitrile (MeCN)–H_2O (13 : 7, v/v)], fraction 4-2 (150 mg) afforded (+)-catechin (**12**). Fraction 5 (20.53 g) was subjected to further chromatography on silica gel (800 g) [EtOAc–MeOH (4 : 1→0 : 1)] which yielded five fractions, fractions 5-1—5-5. Fraction 5-1 (141 mg) was subjected to HPLC [MeOH–H_2O (11 : 9, v/v)] to give aceroside IV (**7**). Chromatography of fraction 5-2 (452 mg) on ODS column (35 g) [MeOH–H_2O (1 : 1→1 : 0)] gave four fractions, fractions 5-2a—5-2d. Further HPLC [MeCN–H_2O (3 : 7, v/v)] was performed on fraction 5-2b (27 mg) which yielded aceroside I (**2**); fraction 5-2c (145 mg) afforded **2**, aceroside III (**3**), and aceroside B_1 (**5**); and fraction 5-2d (210 mg) gave **3**. Percent compositions of individual compounds in the EtOAc-soluble fraction was estimated based on the weight of isolated compounds as: **1** (*ca.* 0.01%), **2** (0.5%), **3** (3.3%), **4** (10.5%), **5** (0.8%), **6** (1.2%), **7** (0.02%), **8** (0.6%), **9** (0.02%), **10** (0.1%), **11** (0.4%), and **12** (0.4%) (refer to Chart 1 for the chemical structures).

Cell Culture B16 murine melanoma cells purchased from Riken Cell Bank (Tsukuba, Japan) were cultured in MEM supplemented with 5% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a humidified CO_2 controlled (5%) incubator.

Determination of Cell Proliferation Using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) based colorimetric assay assessed cell proliferation. After 72 h incubation with test compounds, MTT (100 μl, 5 mg/ml in phosphate-buffered saline) solution was added to the wells. After 3 h of incubation, the medium was removed, and 2-propanol containing 0.04 M HCl was added to dissolve the formazan produced in the cells. The absorbance of each well was then read at 570 nm (reference, 620 nm) by using ELISA Microplate Reader (Labsystem Multiskan MS-UV). The optical density of formazan formed by control cells was used as a reference (assumed to be 100%).

Assay of Melanin Content Test samples dissolved in dimethylsulfoxide (DMSO) were added to the cell culture at final concentration of 25 μg/ml. B16 cells, plated at 2×10^5 cells in a culture dish (60 mmϕ), were pre-incubated for 24 h. After then, the medium was transferred into 5 ml of fresh medium containing the test sample and cultured for 3 d. The cells were harvested by trypsinization. The samples were dissolved in 1 ml of 1 M NaOH containing 10% DMSO, and the amount of melanin was determined spectrophotometrically by absorbance at 420 nm using the ELISA Microplate Reader. The optical density of control cells was assumed to be 100%.

Radical Scavenging Assay The radical scavenging activity of a sample was determined by the DPPH method (slight modification of Terasawa and Yamazaki's method).[9] A sample was dissolved in a DMSO, and then 10 μl of this solution, 200 μl of EtOH, 200 μl of 0.1 M acetate buffer (pH 5.5), and 100 μl of 500 μM DPPH/EtOH were mixed in a test tube. A control was made up with DMSO added instead of the sample solution. The absorbance at 530 nm of the solution was measured 30 min after mixing at room temperature. The radical scavenging activity was calculated using the following equation: % scavenging activity=$[A_{control}-A_{sample}]/A_{control}\times100$. Each sample was measured in triplicate. The 50% inhibitory concentration (IC_{50}; concentration of sample required to scavenge 50% of DPPH radicals) values were determined by the method of probit-graphic interpolation for six concentration levels.

RESULTS AND DISCUSSION

The melanin content was reduced to 34.6% and 62.9% by addition of the *n*-hexane and MeOH extracts (25 μg/ml), respectively, to an incubation medium of the B16 melanoma cell. The *n*-hexane extract inhibited cell proliferation at that concentration whereas the MeOH extract promoted it slightly. The MeOH extract was then fractionated into EtOAc-, *n*-BuOH-, and H_2O-soluble fractions. As compared to the other fractions, the EtOAc-soluble fraction suppressed most significantly the melanin content (58.0% of melanin content at 25 μg/ml) (Table 1). From this fraction we isolated ten diarylheptanoids, **1**—**10**, and two phenolic compounds, **11** and **12**. Six compounds, **1**, **4**, **6**, and **9**—**11**, strongly inhibited melanogenesis in melanoma cell with the melanin content of 7.8—26.1% at 25 μg/ml. Most of the inhibitory activity of these compounds is thought to be due to their cytotoxic action since they reduced significantly cell viability (to 19.2—57.9% of cell viability at 25 μg/ml). Two compounds, **2** and **8**, showed remarkable inhibition of melanogenesis (melanin content 44.4% and 53.2%, respectively, at 25 μg/ml) with no toxicity to the cells because cell viabilities were 100.1% and 108.0%, respectively.

The MeOH extract of the bark of *A. nikoense*, and the EtOAc-, *n*-BuOH-, and H_2O-soluble fractions obtained from the MeOH extract exhibited strong antioxidant activity with IC_{50} values of 1.9—5.0 μg/ml, whereas the *n*-hexane extract showed weak activity (IC_{50} 95.5 μg/ml) (Table 1), on DPPH radical-scavenging assay. This assay has been widely used to measure the radical-scavenging ability of various plant extracts and constituents.[9—11] On evaluation of the DPPH radical-scavenging activity of nine diarylheptanoids, **1**—**7**, **9**, and **10**, and two phenolics, **11** and **12**, isolated from the EtOAc-soluble fraction, four diphenyl ether-type cyclic di-

Table 1. Melanogenesis Inhibitory Activities and Cytotoxicities in B16 Mouse Melanoma Cells, and DPPH Free Radical Scavenging Activities of the Extracts of *Acer nikoense* Stem Barks and the Compounds Isolated from the Extracts

Extract and compound	Melanogenesis inhibitory activity and cytotoxicity		DPPH free radical scavenging activity	
	Mean±S.E. (%)[a]		IC_{50}[c]	
	Melanin content[b]	Cell viability[b]	μg/ml	μM
Control (100% DMSO)	100.0±1.35	100.0±2.50		
n-Hexane extract	34.6±1.80[d]	86.6±1.57[e]	95.5	
MeOH extract	62.9±1.86[d]	115.4±1.82[d]	1.9	
EtOAc-soluble fraction	58.0±2.60[d]	112.8±0.99[d]	4.1	
n-BuOH-soluble fraction	79.5±1.57[e]	121.6±0.68[d]	2.4	
H_2O-soluble fraction	94.4±2.59	113.3±0.78[d]	5.0	
1 Acerogenin A	7.8±0.19[d]	43.6±2.15[d]		40.2
2 Aceroside I	44.4±1.04[e]	100.1±1.05		>200
3 Aceroside III	78.9±5.04	93.3±1.39[d]		44.0
4 (*R*)-Acerogenin B	8.2±0.42[d]	36.0±1.03[d]		40.2
5 Aceroside B_1	28.1±3.87[e]	87.6±1.53[d]		>200
6 Acerogenin D	26.1±0.59[d]	57.9±2.29[d]		40.4
7 Aceroside IV	68.9±8.21	94.7±1.08[d]		> 200
8 Acerogenin M	53.2±2.57[e]	108.0±0.80[d]		
9 Acerogenin K	10.0±0.51[d]	29.6±2.04[d]		>200
10 (−)-Centrolobol	8.0±0.72[d]	19.2±0.59[d]		>200
11 (+)-Rhododendrol	23.4±1.43[d]	47.6±1.29[d]		>200
12 (+)-Catechin	88.3±3.11	108.3±2.67[d]		9.0
Reference compound				
Arbutin	76.4±3.58	102.3±0.81		
α-Tocopherol				27.1

a) Sample concentration determined was 25 μg/ml except for compound **7** which was determined at 12.5 μg/ml. Melanin contents (%) and cell viability (%) were determined based on the absorbances at 420 and 570 nm, respectively, by comparison with those for DMSO (100%). The absorbances at 420 and 570 nm of DMSO were 0.452 and 0.344, respectively. *b*) Values of fourfold experiments. Concentration of DMSO in the sample solution was 5 μl/ml. *c*) Each sample was measured in triplicate. Concentration of DMSO in the sample solution was 20 μl/ml. The 50% inhibitory concentration (IC_{50}) values were determined by the method of probit-graphic interpolation for six concentration levels. *d*) $p<0.001$ by Student's *t*-test compared with the control group. *e*) $p<0.01$ by Student's *t*-test compared with the control group.

arylheptanoids, **1**, **3**, **4**, and **6**, exhibited potent radical-scavenging activities with IC_{50} values of 40.2, 44.0, 40.2, and 40.4 μM, respectively, which being almost comparable with that of a known antioxidant, α-tocopherol (IC_{50} 27.1 μM). Based on the radical-scavenging activities of compounds **2** (IC_{50} >200 μM), **3** (44.0 μM), **5** (>200 μM), **6** (40.4 μM), and **7** (>200 μM), it can be deduced that glycosylation at the phenolic hydroxyl group (compounds **2**, **5**, **7**) reduced significantly the activity, whereas that at the heptyl-chain hydroxyl group (compound **3**) exerted almost no influence on the activity of the diarylheptanoids. This suggests that radical-scavenging activity of the diphenyl ether-type cyclic diarylheptanoids is highly associated with the presence of a free phenolic hydroxyl group. Compound **12** exhibited the most strong radical-scavenging activity (IC_{50} 9.0 μM) among the others tested in this study, and such a high activity of **12** is consistent with that reported recently.[11—13]

Our data indicate that the extracts and some of the diarylheptanoid and phenolic constituents of *A. nikoense* tree bark are useful as skin whitening agents as well as natural antioxidants. Constituents of the *n*-hexane extract, and the *n*-BuOH- and H_2O-soluble fractions from the MeOH extract from the bark of *A. nikoense* are now being investigated.

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